UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE BIOLOGIA ANIMAL

INFLUENCE OF POST-THAW CULTURE PERIOD ON THE DEVELOPMENTAL POTENTIAL OF FROZEN EMBRYOS

ANA MAFALDA LEAL RATO

MESTRADO EM BIOLOGIA HUMANA E AMBIENTE

2009

UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE BIOLOGIA ANIMAL

INFLUENCE OF POST-THAW CULTURE PERIOD ON THE DEVELOPMENTAL POTENTIAL OF FROZEN EMBRYOS

ANA MAFALDA LEAL RATO

MESTRADO EM BIOLOGIA HUMANA E AMBIENTE

2009

DISSERTAÇÃO ORIENTADA

PELO PROFESSOR DOUTOR CARLOS E. PLANCHA

E PELA PROFESSORA DOUTOURA DEODÁLIA DIAS

A apresentação detalhada do tema proposto para Dissertação de Mestrado será feita sob a forma de artigo científico, segundo as regras para autores da revista *Human Reproduction*. Julgo que o conteúdo do trabalho aqui apresentado se enquadra no âmbito particular desta publicação científica, que, para além de possuir um elevado grau de impacto, é amplamente reconhecida pela comunidade Médica e Científica especializada na área da Reprodução Medicamente Assistida.

Influência do período de cultura pós-descongelamento no potencial de desenvolvimento de embriões criopreservados

Resumo

A dualidade entre o benefício das técnicas de Procriação Medicamente Assistida e a produção de embriões excedentários tem desde sempre preocupado não só os profissionais de saúde que lidam diariamente com esta problemática, mas tem também estendido o debate à sociedade em geral. Por vezes esta dualidade ganha tal magnitude, que conduz alguns casais a pesar o desejo de atingir uma gravidez e a inevitabilidade de produzir embriões excedentários. No entanto, ao permitir que os casais beneficiem de mais do que uma transferência embrionária por cada ciclo de punção ovárica, ou seja, ao elevar a taxa de gravidez cumulativa por ciclo de estimulação, os embriões congelados deixaram de ser encarados apenas como um problema, mas sobretudo como parte da solução para os casais inférteis (Gabrielsen *et al,* 2006; Borini *et al,* 2008).

Por outro lado, do ponto de vista da equipa clínica, à medida que as técnicas de PMA se aperfeiçoam, o objectivo de obter uma gravidez *per se*, começa gradualmente a ser substituído pelo objectivo de proporcionar o nascimento de uma criança saudável, tentando evitar as gestações múltiplas, bem como os seus riscos associados. Também nesse sentido a criopreservação de embriões é hoje uma técnica imprescindível nos Centros de PMA, uma vez que possibilita que a um mesmo ciclo de estimulação ovárica correspondam várias transferências de um pequeno número de embriões. No entanto, apesar da tendência para se transferirem menos embriões em cada tentativa, a maioria dos Centros em Portugal ainda não está preparada para propôr a transferência de embrião único (TEU). Apesar das vantagens óbvias da TEU quer em termos de saúde perinatal, quer da gestão dos recursos hospitalares, nomeadamente no que respeita ao internamento decorrente de gravidez múltipla, esta continua a ser uma realidade diária (Sunde, 2007; Shibahara *et al,* 2007). A par da limitação imposta pelos Organismos Públicos ao número de ciclos de PMA a que cada casal tem direito, uma das razões que mais influencia a transferência de mais do que um embrião por tentativa, é a inexistência de um programa de criopreservação de embriões que garanta

uma taxa de implantação e um potencial de desenvolvimento embrionário comparável à dos ciclos a fresco não-electivos.

A melhoria dos protocolos de congelamento/descongelamento é, sem dúvida, uma das abordagens que mais influência terá na decisão da TEU (Macanu *et al,* 2008). Apesar das melhorias ao nível das soluções crioprotectoras e da evolução dos suportes e aparelhos de criopreservação (Borini *et al,* 2008), a maioria das técnicas empregues de forma rotineira não pode evitar a lise de alguns blastómeros, a toxicidade de alguns agentes crioprotectores ou o efeito negativo da desidratação na organização e metabolismo celular.

Se por um lado a capacidade de ultrapassar a maioria destes constrangimentos está dependente da evolução de substâncias e/ou suportes de congelamento, cujo desenvolvimento ultrapassa a esfera de actuação de um laboratório de PMA, existem uma série de outras estratégias, mesmo que empíricas, que podem promover a excelência de um programa de TEC (transferência de embriões congelados) e contribuir, de forma significativa, para a eficiência do processo de criopreservação.

A idade da mulher na altura da punção ovárica, o número médio de embriões transferidos, o estádio de desenvolvimento em que os embriões são transferidos, o número de blastómeros intactos e o reinício da actividade mitótica após descongelamento são tudo factores que podem influenciar um maior ou menor sucesso de um programa de TEC (Van der Elst *et al.,* 1997; Van den Abbeel *et al.,* 2000; Guerrif *et al.,* 2002; Tang *et al.,* 2006; Salumets *et al.,* 2006). A relação entre o dia de desenvolvimento embrionário e a taxa de sucesso após criopreservação é um tema que tem merecido alguma atenção. Se por um lado é aceite que os blastocistos são mais afectados pelo processo de congelamento lento que embriões em estadios de desenvolvimento mais precoce, a decisão de criopreservar embriões ao segundo ou terceiro dia de desenvolvimento ainda é matéria de debate (Salumets *et al.,* 2003; Sifer *et al.,* 2006). No que respeita à selecção dos embriões criopreservados, ocorre uma tendência generalizada para se avaliar a capacidade de clivagem após cerca de 24h de cultura. Pretende-se desta forma, detectar e transferir os embriões que exibam a capacidade de reiniciar a actividade mitótica. Aceita-se que esta informação seja particularmente importante quando se descongela um elevado número de embriões e existe necessidade de distinguir entre

embriões com classificação morfológica idêntica (Van der Elst *et al.*, 1997). Por outro lado, quando a estratégia de descongelamento é mais conservadora, ou seja, quando os embriões são descongelados de forma sequencial, até que se obtenha um número próximo do que será adequado transferir, a necessidade de classificar os embriões com base na actividade mitótica pode ser discutível. No nosso Centro, a avaliação da actividade mitótica com o intuito de seleccionar embriões após descongelamento foi largamente utilizada. No entanto, em 2004, após uma avaliação retrospectiva do programa de transferência de embriões congelados, verificou-se que, na maioria dos casos, os embriões eram pré-seleccionados no próprio dia do descongelamento, independente de reiniciarem ou não a sua actividade mitótica. A partir desse momento, o descongelamento de embriões passou a ser realizado, preferencialmente, no próprio dia da transferência. Durante o período subsequente, a selecção embrionária passou a basear-se principalmente na sobrevivência dos blastómeros e na classificação morfológica.

Aceita-se que os sistemas de cultura embrionária, embora concebidos para se assemelharem ao ambiente da trompa de falópio e intra-uterino, sejam inevitavelmente causadores de algum stress celular (Lane and Gardner, 2005). Isso permite-nos levantar a possibilidade dos embriões criopreservados tolerarem de forma menos eficiente o stress induzido pelo período de cultura pós-descongelamento, o que se poderá reflectir no seu potencial de implantação e desenvolvimento.

Neste estudo retrospectivo, comparámos a influência de um período curto de cultura pós-descongelamento (2-5h) com um período longo (18-24h) no que respeita à taxa de gravidez, implantação e desenvolvimento a termo. Identificámos, pela primeira vez, a associação de um longo período de cultura com a redução do potencial de implantação e desenvolvimento de embriões criopreservados.

No que respeita aos factores passíveis de influenciar as diferenças observadas entre os grupos de cultura, podemos afirmar que o resultado encontrado é independente da idade materna à data da punção ovárica, do número de embriões transferidos, da técnica laboratorial empregue (FIV ou ICSI), do estádio de desenvolvimento embrionário ou da proporção de blastómeros intactos recuperados após descongelamento.

Referências

Borini A, Cattoli M, Bulletti C, Coticchio G. (2008) Clinical efficiency of oocyte and embryo cryopreservation. *Ann N Y Acad Sci*. **Apr,** 1127:49-58.

Gabrielsen A, Fedder J, Agerholm I. (2006) Parameters predicting the implantation rate of thawed IVF/ICSI embryos: a retrospective study. *Reprod Biomed Online*. **12**, 70-6.

Guerif, F., Bidault, R., Cadoret, V., Couet, M.-L., Lansac, J. and Royere, D. (2002) Parameters guiding selection of best embryos for transfer after cryopreservation: a reappraisal. *Hum. Reprod*., **17**, 1321-1326.

Lane, M. and Gardner, D.K. (2005) Understanding cellular disruptions during early embryo development that perturb viability and fetal development. *Reprod. Fertil. Dev*., **17**, 371-378.

Mocanu EV, Cottell E, Waite K, Hennelly B, Collins C, Harrison RF. (2008) Frozen-thawed transfer cycles: are they comparable with fresh? *Ir Med J.*, **101,** 181-4.

Salumets A, Tuuri T, Mäkinen S, Vilska S, Husu L, Tainio R, Suikkari AM. (2003) Effect of developmental stage of embryo at freezing on pregnancy outcome of frozen-thawed embryo transfer. *Hum. Reprod*. Sep;**18**,1890-5.

Salumets, A., Suikkari, A.-M., Mäkinen, S., Karro, H., Roos, A. and Tuuri, T. (2006) Frozen embryo transfers: implications of clinical and embryological factors on the pregnancy outcome. *Hum. Reprod.*, **21**, 2368–2374.

Shibahara H, Hirano Y, Okajima T, Shimada K, Kikuchi K, Suzuki T, Takamizawa S, Suzuki M. (2007) Establishment of criteria for elective single embryo transfer at day 2 or day 3 by analyzing cases with successful implantation of all embryos transferred. *J Obstet Gynaecol Res.* **33**,501-5.

Sifer C, Sellami A, Poncelet C, Martin-Pont B, Porcher R, Hugues JN, Wolf JP. (2006) Day 3 compared with day 2 cryopreservation does not affect embryo survival but improves the outcome of frozen-thawed embryo transfers. *Fertil Steril.* Nov;**86**,1537-40.

Sunde A. (2007) Significant reduction of twins with single embryo transfer in IVF. *Reprod Biomed Online*. **15**, 28-34.

Tang, R., Catt, J. and Howlett, D. (2006) Towards defining parameters for a successful single embryo transfer in frozen cycles. *Hum. Reprod*., **21**, 1179-1183.

Van den Abbeel, E. and Van Steirteghem, A.C. (2000) Zona pellucida damage to human embryos after cryopreservation and the consequences for their blastomere survival and in vitro viability. *Hum. Reprod*., **15**, 373-378.

Van der Elst, J., Van den Abbeel, E.,Vitrier, S., Camus, M., Devroey, P. and Van Steirteghem, A.C. (1997) Selective transfer of cryopreserved human embryos with further cleavage after thawing increases delivery and implantation rates. *Hum. Reprod*., **12**, 1513-1521.

To be submitted to Human Reproduction

Influence of post-thaw culture period on the developmental potential of frozen embryos

Mafalda Rato[1,3] and Carlos E. Plancha[1,2]

[1] Centro Médico de Assistência à Reprodução – CEMEARE, Rua Alfredo Mesquita, 2E, 1600-036 Lisboa

[2] Unidade de Biologia da Reprodução, Instituto de Histologia e Biologia do Desenvolvimento, Av. Professor Egas Moniz, 1649-028 Lisboa, Portugal

[3] To whom correspondence should be addressed

BACKGROUND: Although the cryopreservation of surplus embryos is an established technique, success rates after frozen embryo transfers (FET) are lower than after the transfer of fresh embryos. Apart from slow-freezing related cryodamage, several factors have been identified as major players in the reduction of post-thaw implantation and developmental potential of frozen-thawed embryos. At our center, the interesting observation that embryos thawed on the same day of transfer resulted in a higher pregnancy rate lead us to retrospectively evaluate the influence of the post-thaw culture period on the implantation and developmental potential of cleavage stage embryos. **METHODS:** In this observational study, 305 cycles were allocated to one of two study groups, depending on the post-thaw culture period: 1) 18-24h - the long culture group or 2) 2-5h – the short culture group. Groups were compared regarding implantation rate (IR) and live birth rate *per* embryo (LR/E). **RESULTS:** Implantation and live birth rate *per* embryo were inversely related to the post-thaw culture period, as the FET outcome after a short post-thaw culture period (IR: 10.9%; LR/E: 9.6%) is significantly higher than after a long period (IR: 4.9%; LR/E: 2.9%). The independence of the observed differences was tested for possible confounding factors such as maternal age at oocyte pick-up, number of transferred embryos, developmental day at freezing and blastomere survival after thawing. None of the above mentioned factors was responsible for the observed difference between study groups. No significant advantage was found if only embryos with observed mitotic activity were transferred (IR: 6.5%; LR/E: 5.2%) in comparison to transfers where mitotic resumption was not evaluated (IR: 11.5%; LR/E: 10.0%). After a long post-thaw culture period outcomes were lower (IR: 4.9%; LR/E: 2.9%) than after a fresh transfer (IR: 11.7%; LR/E: 7.4%). However, after a short post-thaw culture period, outcomes become comparable between the fresh and frozen groups (IR: 10.9%; LR/E: 9.6%). **CONCLUSION:** In this study we identified a long post-thaw culture period as being associated with loss of implantation and developmental potential of cleavage stage embryos. Moreover, this study strengthens the efficiency of a frozen-thawed embryo selection based on blastomere survival. Finally, it raises the interesting proposal that, in order to maintain acceptable post-thaw developmental potential, extending embryo culture should be avoided, even to document mitotic resumption.

Key words: embryo cryopreservation / developmental potential / post-thaw culture period / mitotic resumption

Introduction

The cryopreservation of surplus embryos generated in the course of ART (Assisted Reproduction Techniques) is an everyday occurrence in most embryology laboratories and is considered an efficient and safe procedure (Mandelbaum *et al.*, 1998). Apart from long discussed ideological and ethical concerns, embryo freezing is currently viewed as part of the solution for couples undergoing ART. If in one hand it elevates cumulative pregnancy rates (Horne *et al.*, 1997; Bergh *et al.*, 2005), on the other hand, it diminishes the probability of higher order pregnancies, by supporting the transfer of a lower number or even only one embryo (SET - single embryo transfer) at a time (Tiitinen *et al.*, 2001; Tang and Howlett, 2006). Although a well established technique, success rates after FETs

(frozen embryo transfers) are lower than following fresh embryo transfers (Andersen *et al.,* 2009). Several factors contribute to the technique limitations. Some of them relate to the cryoprotective media, others to the cryopreservation devices and/or supports, but no procedure is free of threatening blastomere integrity, embryonic implantation and development potential (Fuller and Paynter, 2004). If the improvement of the freezing technique at the biochemical and biophysical level is beyond the scope of most ART laboratories, there are other valuable strategies that may as well contribute to its efficiency.

The maternal age at OPU (oocyte pick-up), the ART technique used for insemination, the number of transferred embryos, the embryonic developmental day and grade at freezing, the cryosurvival and the post-thaw selection of mitotically active embryos are all factors that have been identified as influencing the efficiency of FETs (Van der Elst *et al.*, 1997; Van den Abbeel *et al.,* 2000; Guerrif *et al.,* 2002; Tang *et al.,* 2006; Salumets *et al.,* 2006). Regarding the freezing day, apart from discouraging results for slow freezing of blastocysts, the survival and pregnancy rates obtained with day 2 *versus* day 3 embryos are still a matter of debate (Salumets *et al.,* 2003; Sifer *et al.,* 2006). As long as post-thaw embryo selection is concerned, there is a wide tendency to perform a 24h culture in order to access blastomere mitotic resumption and consequently, embryonic post-thaw metabolic activity. This information is particularly useful when a high order number of embryos is thawed for each FET attempt (Van der Elst *et al.,* 1997) and it rapidly became a widely employed strategy for post-thaw embryo grading and selection. When embryos are sequentially thawed until the desirable number is reached and the objective is to maximize the number of FETs per each ovarian puncture, the usefulness of such evaluation may be questionable. At our center, since 2000, the evaluation of mitotic resumption was preferentially employed, until a retrospective evaluation of our FET program was performed in 2004. It was noticed that embryos surviving the thawing process were generally the same embryos selected for transfer the following day, irrespective of subsequent mitotic resumption. From that period on, embryo thawing was preferentially performed on the same day as transfer, and embryo selection strategy was based mostly on survival and morphological grading.

It is accepted that embryo culture systems, although aiming to mimic the physiological environment, do not replace it completely (Lane and Gardner, 2005). This raises the possibility that post-thaw culture could provide insufficient support to the less fit frozen-thawed embryos, diminishing their implantation and developmental potential. In this study we compared a short (2-5h) with a long (18-24h) post-thaw culture period upon the pregnancy rates, implantation rates and development to term. We identified a long post-thaw culture period as being associated with a loss of implantation and embryo developmental potential. In contrast, a short post-thaw culture period was shown to associate with increased implantation and developmental potential of frozen cleavage stage embryos.

Materials and methods

Patient inclusion and study period

All procedures evaluated were performed at Centro Médico de Assistência à Reprodução (CEMEARE) in Lisbon, Portugal. Patients included in this study underwent IVF/ICSI cycles with cryopreservation of supernumerary embryos and initiated a FET cycle between May 2001 and December 2008. Patients were included as long as they had at least, one viable embryo transferred - defined as having retained ≥50% of their initial number of blastomeres intact upon-thawing. The post-thaw culture period did not exceed 24h.

In this retrospective study, data from a total of 332 FET cycles was reviewed and 305 cycles were included. Two main periods concerning two different post-thaw and embryo selection strategies were traversed. The first period extended mainly from May 2001 to June 2005 and coincided with

the established tendency to select embryos after evaluation of their mitotic activity. The second period extended mainly from July 2005 to December 2008 and significantly represented our new tendency to reduce the culture period between thaw and transfer, resulting on a greater emphasis on embryo survival.

Finally, FET outcome was compared with the outcome of 238 non-elective fresh transfers occurring between October 2000 and December 2007.

IVF/ICSI procedures

Patients undergoing IVF/ICSI cycles were carefully evaluated in terms of infertility aetiology and assigned for either IVF or ICSI procedures according to strict laboratory criteria, that is, ICSI was only performed in cases of severe male factor, independent of the number of oocytes at OPU or woman's age.

Ovarian stimulation was preceded by a pituitary down-regulation using, or not, a GnRH agonist (Decapetyl, Ipsen Pharma Biotech, Signes, France) or antagonist (Orgalutran, Organon/Schering-Plough, UK; Cetrotide, Serono, London, UK). When suppression was accomplished, human menopausal or recombinant FSH was administered by transdermal injection. Serum estradiol levels and follicle count and diameter were evaluated periodically in order to allow cycle monitoring. When two or more follicles reached the size of ≥18mm, hCG (Pregnyl, Organon/Schering-Plough, Oss, Holand; Ovitrelle, Serono, London, UK) was administered in order to induce oocyte maturation and oocyte-cumulus-complex (COC) expansion. Transvaginal follicle aspiration was performed 36-38h after hCG
administration and COCs were identified in the laboratory, washed in Flushing medium with and without heparin (all culture media from MediCult, Jyllinge, Denmark) and incubated in IVF medium for 2-5h before insemination by either IVF or ICSI.

Incubation was carried under mineral oil in a humidified, 6% CO_2 controlled atmosphere, at 37^{o}C. All bicarbonate-buffered media were pre-equilibrated in this atmosphere overnight. Fertilization was evaluated 16-19h after insemination and confirmed by pronuclei and polar body observation. For most embryos, culture was performed using sequential media. Accordingly, zygotes and day 2 embryos were cultured in ISM1 medium and transferred to ISM2 medium at day 3.

Embryos were scored every day for blastomere number, degree of fragmentation and blastomere cleavage plane according to the following criteria: 1.0) embryos with blastomeres resulting from a correct cleavage plane with no fragmentation; 2.0) embryos with blastomeres resulting from a slight deviation from the correct cleavage plane with no fragmentation; 2.1) embryos with ≥10% fragmentation; 2.2) embryos with >10% ≤20% fragmentation; 3.1) embryos with >20% <50% fragmentation; 3.2) embryos with ≥50% fragmentation. The best embryos were selected for fresh embryo transfer. Surplus embryos up to grade 2.2 were considered suitable for cryopreservation.

Cleavage stage embryo slow freezing and rapid thawing

According to the slow freezing protocol, embryos were sequentially equilibrated at room temperature in a HEPES-buffered medium containing cryoprotectants up to a final concentration of 1.5M 1,2-propanediol (PROH) and 0.1M sacarose (Medicult freezing pack,. Embryos were loaded into ministraws (CryoBioSystems, France) up to a maximum of three per straw and cooled using a programmable freezer (MiniCool, AirLiquid, France). The cooling program started at 20 ºC and ran as follows: temperature was taken to -7^{o}C at a rate of 2^{o}C/min and manual seeding was performed by touching the extremes of the straw with a nitrogen-cooled forceps. The cooling program slowly decreased the temperature at a 0.3^{o}C/min to -30^{o}C. A final and very fast drop to -150^{o}C was followed by a temperature decrease of 50^{o}C/min. Straws were then plunged into liquid nitrogen and stored until the moment of thawing.

Rapid thawing was performed by quickly placing the straws on hand for a minimum of 30s. Embryos were then discharged to a 4-well dish and washed from the cryoprotectors at room temperature, using sequential thawing media (Medicult thawing pack).

According to their developmental day upon freezing, embryos were transferred back to the appropriate culture medium (ISM1 or ISM2), checked for blastomere survival under an inverted microscope at a 200x magnification and incubated until transfer.

Straws were thawed one by one until the ideal number of surviving embryos was reached. Since most straws carried more than one embryo, sometimes this number was slightly over passed.

Embryo selection strategy based on evaluation of mitotic activity – long culture group

During the period from May 2001 to June 2005, the most common procedure was to thaw the embryos the day before the transfer and culture them for a long 18-24h period. Both upon thawing and before transfer embryos were classified in terms of blastomere number and morphology. This methodology allowed the evaluation of mitotic activity. Together with information on blastomere loss, embryos were classified as: Top quality) if no blastomere loss occurred and mitosis resumed, Good quality) if some blastomeres were lost and mitosis resumed, Transferable) if none or some blastomere loss occurred but mitosis didn't resume and Non-transferable) if more than half the initial blastomere number was lost. During this study period, providing that viability was maintained, mitotic resumption was considered to be of great importance regarding embryo selection.

Embryo selection strategy based on survival – short culture group

During the period from July 2005 to December 2008, there was an increased tendency to thaw the embryos on the same day of transfer and culture them for a short 2-5h period before transfer. The thawing procedure remained unchanged. Blastomere number and embryo morphology were recorded and embryos were classified as: Top quality) if no blastomeres were lost, Good quality) if at least half the initial number of blastomeres was maintained and Non-transferrable) if more than half the initial number of blastomeres were lost. During this study period, embryo survival was considered to be the most important factor for embryo selection.

Uterine transfer of frozen-thawed embryos

Embryo transfer back to the uterus was performed following endometrium preparation. Accordingly, the supplementation protocol was initiated, during the late lutheal phase, with a Leucoprolide injection (Decapetyl depot 0,1mg/ml, Ipsen Pharma Biotech, Signes, France) - a GnRH agonist, for hypothalamic suppression. This downregulation of endogenous gonadotrophins was followed by the administration of 17β-estradiol (Estrophem, Novo NordisK) in order to mimic the natural occurring estrogens. This supplementation started at the first or second day of the reproductive cycle at an oral dose of 2mg/day until the ninth day of the cycle, continued during three more days at a dose of 4mg/day and was increased to 6mg/day until the endometrium reached 8mm thick and the serum estradiol levels reached ≥150mg/dl. At this point, embryo transfer was performed.

Before transfer, embryos were rechecked for viability, placed in pre-equilibrated Universal Transfer Medium (UTM) and loaded into an appropriate catheter. Upon thawing, while some embryos remained intact, some lost up to 50% of the initial number of blastomeres, resulting in three types of transfer: type A) only intact embryos were transferred, type B) intact and partially damaged embryos were transferred, type C) only partially damaged embryos were transferred.

Regarding mitotic activity, whenever appropriate, transfers were classified as: type D) only embryos with observed mitotic resumption were transferred, type E) embryos with and without observed mitotic resumption were transferred and

type F) only embryos without observed mitotic resumption were transferred. In most cases of the short culture group, mitotic activity was not evaluated.

From this stage on, progesterone (Utrogestan, Jaba Recordati, Sintra, Portugal) was added to the previous therapeutic scheme at a dose of 800mg/day, vaginal application, during three or four days. Serum βhCG concentration was evaluated 12 days to 14 days after transfer. Pregnancy was considered to have occurred when a value ≥ 10pg/ml was detected. In these cases, estrogen and progesterone supplementation carried on until the twelfth week of gestation. Clinical pregnancy was defined by the presence of a gestational sac with a fetal heartbeat on ultrasound examination at 6 weeks of gestation.

Evaluation of success parameters and statistical analysis

During the course of this study, several parameters were used to define success or failure of the embryo transfers. As above mentioned, embryos were considered to have survived freezing whenever they retained at least half the initial number of blastomeres upon thawing. Blastomeres did not survive whenever they were considered to be lysed. Survival rate was defined as the number of viable embryos *per* number of thawed embryos. Pregnancy rate was defined as the number of pregnancies *per* number of transfers. Implantation rate was defined as the number of gestational sacs *per* number of transferred embryos. Abortion rate was defined as the number of non-evolutive gestational sacs *per* number of gestational sacs observed. Twin pregnancy rate was defined as the number of twin pregnancies *per* total number of pregnancies. Take-home baby rate was defined as the number of babies born *per* number of transfers. Live birth rate *per* embryo was defined as the number of babies born *per* embryo transferred. The birth of a baby, at least, was considered the highest overall success of the technique.

Statistical analysis was performed using STATISTICA 8.0. Depending on the variables, ANOVA, t-Student or non-parametric test for independent variables were used. Differences were considered significant if $p < 0.05$.

Results

Characterization of the study groups – frozen and fresh (non-elective) embryo transfers

The two FET groups studied were characterized in terms of maternal age at OPU, embryo developmental day at freezing, number of thawed, surviving and transferred embryos, ART technique (IVF/ICSI), and extent of blastomere loss and mitotic resumption of transferred embryos. Both FET groups were also compared to the fresh, non-elective transfers performed during the correspondent time period. The mean ± SD of maternal age at OPU of the patients included in the long and short culture groups and in the fresh group was equivalent (32.3 ± 4.2; 33.5 ± 4.2 and 34.8 ± 4.6) (Table I). The mean number of transferred embryos was also found to be equivalent in all studied groups (2.4 ±0.7; 2.6 ±0.8 and 2.2 ±0.9) (Table I).

The distribution of frozen embryo transfers in relation to the fertilization technique (IVF/ICSI), developmental day at freezing, blastomere loss and post-thaw mitotic resumption was also evaluated. Regarding the freezing day, the number of FETs performed with day 2 and day 3 embryos was not equivalent between groups, as 75% of transfers from the long culture group occurred with embryos cryopreserved on day 2 and 25% on day 3, and in the short culture group, 40.9% of transfers were performed with embryos frozen on day 2, 52.9% on day 3 and 6.2% on day 4 (Table II). Regarding the laboratory technique used for oocyte insemination, the proportion of embryos resulting from IVF (n=38/80; n=109/225) and ICSI (n=37/80; n=105/225) was not statistically different in both FET groups. The proportion of transfers using only intact embryos – type A transfers (n=19/80; n=49/225), and only partiallydamaged – type C (n=20/80; n=84/225) embryos, in both long and short culture groups was

Table I – Outcome of embryo transfers following a different post-thaw culture period and following a fresh cycle with non-elective transfer.

	Long culture group (18-24h)	Short culture group (2-5h)	Fresh non-elective transfers
No. Embryo transfers	80	225	238
mean age (± SD) at OPU	32,3 (± 4.2)	33,5 (± 4.2)	34,8 (± 4.6)
No. Thawed embryos	341	883	-
mean (± SD)	4,3 (± 1.9)	3,9 (± 1.7)	-
No. Surviving embryos	242	624	-
mean (± SD)	3,0 (±1.3)	2,8 (±1.1)	-
% Surviving embryos	71,0	70,7	-
No. Transferred embryos	205	530	512
mean (± SD)	2,6 (± 0.8)	2,4 (± 0.7)	2,2 (± 0.9)
No. Clinical pregnancies	10	48	51
Pregnancy rate (%)	12,5	21,3	21,4
Twin rate *per* Clinical pregnancy (%)	0	10 (20,8)	8 (15,7)
No. Gestational sacs	10	58	60
Implantation rate (%)	4,9[a,b]	10,9[a]	11,7[b]
No. Non-evolutive gestational sacs	4	7	19
Abortion rate(%)	40,0[c]	12,1[c,d]	31,7[d]
No. Deliveries	6	42	33
mean age (± SD) of women at OPU	30,8 (± 4,6)	31,8 (± 4,5)	33,6 (± 3.1)
mean number (± SD) of transferred embryos	2,8 (± 0,7)	2,4 (± 0,6)	2,4 (± 0,9)
No. Children born	6	51	38
Take-home baby rate (%)	7,5[e,f]	22,7[e]	16,0[f]
Live birth rate per embryo (%)	2,9[g,h]	9,6[g]	7,4[h]

Values with the same superscript are statistically different; $p<0.05$

also not statistically different in both FET groups. The efficiency of the freeze/thaw procedure, given by the number of surviving embryos *per* embryos thawed, also remained unchanged for the long and short culture groups (71.0%; 70.7%) (Table I).

As expected, the number of transfers where all embryos resumed mitosis – type D, was significantly higher in the long (n=34/80) than in the short culture group (n=10/225) (Table II), as only a small portion of embryos resumed mitosis within the short period of culture (n=200/225).

Success rates after long or short post-thaw culture

The outcome of each of the post-thaw culture groups followed an increasingly more objective evaluation, going from the determination of the pregnancy rate to the birth of a healthy baby. By comparing the two culture strategies, both the pregnancy and take-home baby rates observed were higher for the short culture group (21.3% and 22.7%) than for the long culture group (12.5% and 7.5%), however this difference did not reach statistical significance regarding pregnancy rate. The twin pregnancy rate in the short culture group was 20.8% while in the long culture group no twin pregnancies occurred. Abortion rates were significantly higher in the long (40.0%) than in the short culture group (12.1%). Going into more demanding analysis of success, both implantation rate and live birth rate *per* embryo were found to be significantly higher in the short (10.4% and 9.6%) than in the long culture group (4.9% and 2.9%) (Table I).

In order to evaluate the difference between FET and fresh transfers, outcomes after a long and short culture period were compared to fresh, non-elective transfers. Implantation and live birth rate *per* embryo where significantly lower in the long culture (4.9% and 2.9%) than in the fresh group (10.5% and 6.6%). Interestingly, no difference was found after the non-elective transfer of fresh embryos and embryos derived from the short culture group (12.1% and 9.6%) (Table I).

Influence of the freezing day, blastomere loss and visualization of mitotic resumption on success

To access the influence of the embryonic developmental stage at freezing, FET outcome concerning implantation and live birth rate *per* embryo were evaluated in each group. No relation was found between a specific day of embryonic development and a better FET outcome when such comparison was performed inside the study groups. In order to evaluate the influence of the post-thaw culture duration on the specific freezing day (day 2 or day 3), the corresponding implantation rate and live birth rate *per* embryo were compared between groups. A significantly higher implantation rate and live birth *per* embryo was observed for embryos frozen at day 2, when cultured for a short period (12.4% and 10.6%) rather than for a long period of time (3.8% and 1.9%) (Table III). The outcome of day 3 embryos does not seem to be influenced by the different post-thaw culture periods.

In order to evaluate the influence of the post-thaw culture period on the embryonic developmental potential of fully intact or partially damaged embryos, type A and C transfers were compared inside and between groups. In the first analysis, and although no babies were born after type A transfers in the long culture group, this difference was not significant in terms of implantation and live birth rate *per* embryo. In the second analysis, implantation and live birth rate per embryo were higher in the short than in the long culture group for both transfer types (type A: 10.4% and 10.4% vs 2.3% and 0.0%; type C: 11.4% and 9.8% *vs* 7.8% and 5.9%), but no significant differences were found (Table IV).

Table II - Distribution of FETs regarding laboratory technique, embryonic developmental day at freezing, blastomere loss and mitotic resumption.

	Long culture group (18-24h)	Short culture group (2-5h)
	N (%)	N (%)
No. Frozen Embryo Transfers (Total):	80	225
derived from IVF	38 (47.5)	114 (50.7)
derived from ICSI	42 (52.5)	111 (49.3)
No. Transfers with:		
day 2 embryos[a]	60 (75)	92 (40.9)
day 3 embryos[a]	20 (25)	119 (52.9)
day 4 embryos[a]	0 (0)	14 (6.2)
No. Transfers regarding blastomere loss:		
Type A	19 (23.8)	49 (21.8)
Type B	41 (51.3)	92 (40.9)
Type C	20 (25.0)	84 (37.3)
No. Transfers regarding mitosis:		
Type D[b]	34 (42.5)	10 (4.4)
Type E[b]	29 (36.3)	13 (5.8)
Type F[b]	12 (15.0)	2 (0.9)
Not evaluated[b]	5 (6.3)	200 (88.9)

Variables with the same superscript have a significantly different distribution in the long and short culture groups. $p<0.05$

The relation between observed mitotic activity and FET outcome was also studied inside and between post-thaw culture groups. In the first analysis, where only data from the long culture group was considered, no statistical association was found between type D or F transfers (after which no babies were born) and a higher implantation (6.5%; 3.8%) or live birth rate *per* embryo (5.2%; 0.0%). The comparison between groups was only performed for type D transfers from the long culture group and transfers from the short culture group where mitosis was not evaluated. No difference was found for implantation (6.5% *vs* 11.5%) or live birth rate *per* embryo (5.2% *vs* 10.0%) (Table V). In all the above mentioned analysis, groups were equivalent regarding mean maternal age at OPU and number of transferred embryos.

Table III - Influence of the embryonic developmental day at freezing on the implantation and live birth rates following different post-thaw culture periods.

	Long culture group* (18-24h)		Short culture group* (2-5h)		
	day 2	day 3	day 2	day 3	day 4
Age at OPU (mean ± SD)	32.8 (± 4.0)	30.8 (± 4.8)	33.7 (± 4.4)	33.3 (±4.5)	34.3 (±4.3)
No. Transferred embryos	158	47	217	274	36
(mean ± SD)	2.6 (± 0.8)	2.4 (± 0.7)	2.4 (± 0.6)	2.3 (± 0.8)	2.6 (± 0.6)
No. Gestational sacs	6	4	27	25	6
Implantation rate (%)	3,8[a]	8,5	12,4[a]	9,1	16,7
No. Children born	3	3	23	22	6
Live birth rate per embryo (%)	1,9[b]	6,4	10,6[b]	8,0	16,7

*Inside groups no differences were found between embryo developmental stages at freezing.
Values with the same superscript are statistically different; $p<0.05$

Table IV - Influence of blastomere loss on the implantation and live birth rates following different post-thaw culture periods.

	Type of transfer* Long culture group (18-24h)			Type of transfer* Short culture group (2-5h)		
	A	B	C	A	B	C
Age at OPU (mean ± SD)	32.1 (± 4.7)	32.2 (± 3.9)	32.6 (± 4.8)	33.8 (± 4.1)	33.5 (± 4.2)	33.5 (±4.9)
No. Transferred embryos	44	106	51	106	240	184
(mean ± SD)	2.3 (± 0.8)	2.7 (± 0.7)	2.6 (± 0.8)	2.2 (± 0.7)	2.6 (± 0.6)	2.2 (± 0.7)
No. Gestational sacs	1	5	4	11	26	21
Implantation rate (%)	2,3[a]	4,7	7,8	10,4[a]	10,8	11,4
No. Children born	0	3	3	11	22	18
Live birth rate per embryo (%)	0,0[b]	2,8	5,9	10,4[b]	9,2	9,8

Type of transfer: type A - only intact embryos were transferred; type B - intact and partially damaged embryos were transferred; type C - only partially damaged embryos were transferred.
*Inside groups no differences were found between the types of transfer.
Values with the same superscript are statistically different; $p<0.05$

Table V - Influence of mitotic resumption on the implantation and live birth rates following different post-thaw culture periods.

	Type of transfer* Long culture group (18-24h)			Type of transfer* Short culture group (2-5h)		
	D	E	F	(Not evaluated)	D**	E**
Age at OPU (mean ± SD)	32.0 (± 4.7)	32.2 (± 3.9)	33.9 (± 4.1)	33.3 (±4.6)	37.7 (± 3.6)	34.1 (± 4.5)
No. Transferred embryos	77	82	26	480	18	32
(mean ± SD)	2.3 (± 0.8)	2.9 (± 0.6)	2.4 (± 0.9)	2.4 (± 0.7)	1.8 (± 0.8)	2.5 (± 0.5)
No. Gestational sacs	5	3	1	55	1	2
Implantation rate (%)	6,5[†]	3,7	3,8	11,5[†]	5,6	6,3
No. Children born	4	1	0	48	1	2
Live birth rate per embryo (%)	5,2[‡]	1,2	0,0	10,0[‡]	5,6	6,3

Type of transfer: type D - only embryos with observed mitotic resumption were transferred; type E - embryos with and without observed mitotic resumption were transferred; type F - only embryos without observed mitotic resumption were transferred.
*Inside groups no differences were found between the types of transfer.
** A relatively small number of embryos resumed mitosis during the short post-thaw period.
†, ‡ Differences were not significant between groups; $p>0.05$

Discussion

In this observational study, 305 FET cycles, resulting in 732 embryos transferred, were allocated to one of two working groups, depending on the post-thaw culture period: a 18-24h period, termed the long culture group or a 2-5h period, termed the short culture group. In this study it was found that a short post-thaw culture period was associated with higher implantation and live birth rate *per* embryo. To our knowledge, this is the first study to identify a long post-thaw culture period associated with implantation and developmental potential loss. Moreover, this study strengthens the efficiency of a frozen-thawed embryo selection approach based on survival (Van del Abbeel *et al.*, 1997; El-Toukhy *et al.*, 2003). Such selection criteria may be more adjusted to the everyday reality of embryology laboratories that follow a step-by-step, conservative thawing policy, intended to maximize the number of transfers *per* OPU.

Although the alteration of the post-thaw culture period was mainly driven by a retrospective evaluation of our FET program in 2005, such modification was not performed without theoretical fundament. Embryo culture, although aiming to mimic the fallopian tube and uterine environment, always implies the induction of some related stress (Summers and Biggers, 2003; Lane and Gardner, 2005; Leese *et al.*, 2008). As stated by Leese *et al.*, 2007, "viable embryos may be better equipped to contend with damage to the genome, transcriptome and proteome". It is thus reasonable to speculate this may be particularly pertinent following cryopreservation. If this is the case, a protocol diminishing post-thaw culture duration could be advantageous.

Embryos exhibit higher implantation and developmental potential with shorter post-thaw culture period

As pregnancy rate does not discriminate between implantation of one or more embryos, and is still an intermediate outcome, implantation rate and live birth rate *per* transferred embryo, although more demanding, are clearly a more informative means of comparison between groups regarding embryo developmental potential. As so, although pregnancy rates were not statistically different between groups, implantation and live birth rates *per* embryo were significantly higher after the short (10.9%; 9.6%) than the long culture group (4.9%; 2.9%), emphasizing the biological impact of the two culture periods. Moreover, the abortion rate was significantly higher when embryos were cultured for a longer period (40% *vs* 12.1% in the short culture group). Regarding twin pregnancy, despite it can be regarded as success or nonsuccess, its specific allocation to the short culture group (20.8%) and total absence in the long culture group (0.0%) confirms a higher implantation and developmental potential with the first group referred. All these results are in accordance with the interpretation that embryos exhibit higher implantation and developmental potential if briefly maintained in culture after thawing. This observation is in line with the hypothesis that cryopreserved embryos may be less able to cope with and adapt to sub-optimal environments such as the presently available culture media and conditions.

Maternal age at OPU and number of transferred embryos do not account for the observed differences between study groups

Regarding confounding factors such as maternal age at OPU and number of transferred embryos, the two accepted most important causes of bias in intra- and inter-assay comparisons, the results allow clear conclusions in our study. Although the mean maternal age at OPU was slightly higher and the mean number of transferred embryos was slightly lower in the short (33.5 ± 4.2; 2.4 ± 0.7) *versus* the long culture group (32.3 ± 4.2; 2.6 ± 0.8), no statistical significance was found. Importantly, if such difference had been found to be significant, it would have no influence on our main observation, as the most successful group had the highest mean age and lowest mean number of embryos transferred, factors that we would expect to negatively influence the outcome.

Influence of embryonic developmental day and embryo grading at freezing, and blastomere loss and mitotic resumption after thawing, on the observed differences between study groups

Many additional factors influencing FET success have been described and their contributions to the cryopreservation field are today variably accepted. The two mostly referred embryological factors at freezing are the embryonic developmental day (Salumets *et al*., 2003; Sifer *et al*., 2006) and embryo grading (Karlstrom *et al*., 1997). The two most discussed embryological factors after thawing are the extent of embryo damage (Van del Abbeel *et al.*, 1997; Guerif *et al.*, 2002) and the visualization of mitotic resumption (Van der Elst *et al.*, 1997; Ziebe *et al.*, 1998).

1) Developmental day at freezing

The developmental day at which embryos were cryopreserved followed no specific criteria and, depending on laboratory schedule, embryos where frozen on the same or the next day following fresh transfer. Nevertheless, the distribution of embryos frozen at day 2, day 3 or day 4 was not proportional between the two study groups. According to previous reports, there is no established agreement on whether or not a specific freezing day is related to a better outcome following FET (Salumets *et al.*, 2003; Sifer *et al.*, 2006). In order to exclude the influence of this possible confounding factor on our main observation, implantation and live birth rate *per* embryo were compared inside groups. No specific day was found to preferentially correlate to a better outcome, indicating that our main finding is independent of embryonic developmental day at freezing. Recently, results in favor of day 3 embryos have been published (Sifer *et al.*, 2006). In the above mentioned publication day 3 embryos are thawed a few hours before transfer while day 2 embryos are cultured overnight before transfer. Interestingly, in order to discuss their conflicting result regarding other reports (Salumets *et al.*, 2003), authors propose that overnight post-thaw culture may negatively influence the outcome of day 2 embryos. Data from the present study seem to correlate such hypothesis. We have observed a fairly lower implantation rate for day 2 embryos in the long, in relation to the short culture group (3.8% vs 12.4%), which leads us to propose that day 2 embryos could be more vulnerable to post-thaw culture stress than day 3 embryos. However, only a prospective study could give us a more detailed insight on this subject.

2) Embryo grading at freezing

As far as embryo grading before freezing is concerned, we did not find any correlation with embryo survival or FET outcome. We can only state that laboratory criteria for cryopreservation remained unchanged during the whole study period. Specifically, embryos were considered suited for cryopreservation if they presented ≤ 20% fragmentation. In this sequence, we can only hypothesize that embryo grading is expected to be randomly equivalent in both groups. Although not expectable, a better grading in a specific group could positively influence its outcome (Van del Abbeel *et al.*, 1997).

3) Blastomere survival after thawing

On the topic of blastomere survival after thawing, and although contradictory reports have been published over the years (Hartshorne *et al.*, 1990; Van del Abbeel *et al.*, 1997; Archer *et al*.,2003), there is a general agreement that FET outcome benefits from the transfer of intact post-thaw embryos (Salumets *et al.*, 2006). In our results, in order to exclude such influence from our main observation, the outcome of type A and C transfers on each culture group was analyzed and found to be equivalent. However, this observation needs to be carefully interpreted, regarding the low number of embryos assessed, in comparison to large series published (El-Toukhy *et al.*, 2003). Beyond the prognostic value of blastomere loss, the most significant finding relates to the difference between type A transfers from the long and short culture groups. When only intact embryos were transferred, implantation and live birth rate *per* embryo were significantly higher if embryos were briefly cultured (10.4% and 10.4%

vs 2.3% and 0.0% in the long culture group), once again pointing to a negative influence of the post-thaw culture period on the embryonic developmental potential. As this difference was not detected for type C transfers, its exact significance remains an open issue. A possible explanation could rely on the documented toxic effect induced by lysed blastomeres (Rienzi *et al.,* 2002; Nagy *et al.,* 2005), that could increase with extended culture period.

4) Mitotic resumption after thawing

Concerning the concept of embryo selection based on visualization of post-thaw mitotic activity, although some previous reports seem to demonstrate that post-thawed cleaved embryos hold a higher developmental potential than non-cleaved ones (Van der Elst *et al*., 1997; Ziebe *et al.,* 1998; Salumets *et al.,* 2006), such concept must be used with caution regarding each one's everyday practice. Following a retrospective evaluation of the FETs performed from May 2000 to June 2005, we came to the conclusion that in 80% of FETs included in long culture group (n=64/80), embryo selection did not benefit from evaluation of mitotic resumption, that is, embryos could have been selected in the same day of thawing (data not shown). Also in our study, during the period where the embryo selection strategy was based on cleavage observation, we found no statistical advantage in transferring only cleaved embryos (implantation and live birth rates *per* embryo after type D *vs* type E transfers: 6.5% and 5.2% *vs* 3.7% and 1.2%). We believe these results are comparable to the rates found by Van der Elst *et al*., 1997 for the same type of transfers (7.7% and 6.5% *vs* 2.9% and 0.6%), but possibly due to a larger number of embryos evaluated, these authors found this difference to be significant. Nonetheless, these studies are not quite comparable, as we perform non-elective FETs in the majority of cases, in contrast to the published elective approach (Van der Elst *et al*., 1997). When the relevance of mitotic activity is to be discussed we should not underestimate the possible influence of the *in vitro* long culture on the embryonic developmental potential. As an example, it was published some years ago that ICSI derived embryos had a decreased potential to develop to blastocyst *in vitro* when compared to their IVF counterparts (Griffiths and Herbert, 2000). Nonetheless, this difference could not be detected if embryo replacement was to be performed in earlier embryonic stages (Plachot *et al.,* 2002), thus depicting a "culture effect". The concept that must be strengthened from all of these results is that inability to develop *in vitro* is no evidence of developmental incompetence *in vivo*. Our results are in favor of such observation, as no differences (regarding implantation rate and live birth *per* embryo) were found after transfer of cleaved embryos (the classically considered "best embryos") (6.5% and 5.2%) and embryos selected based on survival only (short culture group) (11.5% and 10.0%).

Comparison of frozen-thawed and fresh, non elective transfers regarding implantation and developmental potential

To give additional credit to our interpretation of a causal effect between post-thaw embryo culture duration and developmental potential, FET outcomes from both working groups were compared to non-elective fresh transfers. In 2000, Edgar *et al.* published a quantitative analysis of the impact of cryopreservation on the implantation potential of early stage embryos. In this large study series, fresh and frozen embryos of exactly the same quality were compared using a case-match analysis. In this study, the authors concluded that, as long as no damage occurred, cryopreservation had no impact on embryo developmental potential. Facing the inability to perform such comparison, in our study we turned to a retrospective comparison of FETs to non-elective fresh transfers. In these fresh transfers, we transfer the embryo(s) we actually have, and there is no place to select embryos for transfer. We consider this non-elective scenario to roughly resemble the circumstances of most FETs. By doing so, we have found that the developmental potential of embryos with signs of mitotic resumption in one or more blastomeres

(2.9%) was significantly lower than the one resulting from these fresh transfers (6.6%). On the contrary, the reduction of the post-thaw culture period was able to maintain such potential (slightly surpassing it: 9.6% *vs* 6.6%, p=0.01) at the fresh embryos level. This observation is rather encouraging, as it illustrates a neutral impact of a short post-thaw culture period on the implantation and developmental potential of frozen embryos. It also supports the hypothesis that cryopreserved embryos may be less able to adapt to long *in vitro* sub-optimal settings.

Final considerations and conclusion

Although we all agree that the best outcome achieved after embryo cryopreservation will result from embryos with a high score before freezing, who retain their initial number of blastomeres and who maintain the potential for resuming mitotic activity during the post-thaw culture period, this is a far less common situation. In turn, most FETs are performed with second choice, partially damaged embryos, some with no observed mitotic activity and even so, although at a lower rate, result in live births. Our present study raises the interesting proposition that it may not be desirable to actually see resumption of mitotic activity in some blastomeres of thawed embryos. We believe it is important to recognize that a prolonged post-thaw culture period can be causing a decrease in the implantation and developmental potential of thawed cleavage stage embryos. In order to validate our hypothesis, it would be essential to perform a prospective randomized study.

Acknowledgments

The authors would like to thank Prof. Dr. Rui Figueira and Dr. Augusto Eusébio for detailed statistical discussion. They would also like to thank CEMEARE staff, especially Dr. Sara Pimentel, for encouraging support and thoughtful discussion of results.

References

Andersen, A.N., Goossens, V., Bhattacharya, S., Ferraretti, A.P., Kupka, M.S., Mouzon, J., Nygren, K.J. and The European IVF monitoring (EIM) Consortium, for the European Society of Human Reproduction and Embryology (ESHRE) (2009) Assisted reproductive technology and intrauterine inseminations in Europe, 2005: results generated from European registers by ESHRE. *Hum. Reprod.*,.**24**, 1267–1287.

Archer J., Gook D.A., Edgar D.H., (2003) Blastocyst formation and cell numbers in human frozen–thawed embryos following extended culture. *Hum. Reprod.*, **18**, 1669-1673.

Bergh, C., Josefsson, B., Nilsson, L. and Hamberger, L. (1995) The success rate in a Swedish *in-vitro* fertilization unit: a cohort study. *Acta Obstet. Gynecol. Scand.*, 74, 446-450.

Brison, D.R., Houghton, F.D., Falconer, D., Roberts, S.A., Hawkhead, J., Humpherson, P.G., Lieberman, B.A. and Leese H.J. (2004) Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover. *Hum. Reprod.*, **19**, 2319-2324.

Edgar, D.H., Bourne, H., Speirs, A.L. and McBain J.C. (2000) A quantitative analysis of the impact of cryopreservation on the implantation potential of human early cleavage stage embryos. *Hum. Reprod*., **15,** 175-179.

El-Toukhy, T., Khalaf, Y., Al-Darazi, K., Andritsos, V., Taylor, A. and Braude, P. (2003) Effect of blastomere loss on the outcome of frozen embryo replacement cycles. *Fertil. Steril.*, **79**, 1106-1111.

Fuller, B. and Paynter, S. (2004) Fundamentals of cryobiology in reproductive medicine. Reprod. BioMed. Online, **9**, 680-691.

Guerif, F., Bidault, R., Cadoret, V., Couet, M.-L., Lansac, J. and Royere, D. (2002) Parameters guiding selection of best embryos for transfer after cryopreservation: a reappraisal. *Hum. Reprod*., **17,** 1321-1326.

Griffiths, T.A., Murdoch, A.P. amd Herbert, M. (2000) Embryonic development in vitro is compromised by the ICSI procedure. . *Hum. Reprod*.**15**, 1592-1596.

Hartshorne, G.M., Wick, K., Elder, K. and Dyson, H. (1990) Effect of cell number at freezing upon survival and viability of cleaving embryos generated from stimulated IVF cycles

Horne, G., Critchlow, J.D., Newman, M.C., Edozien, L., Matson, P.L. and Lieberman, B.A. (1997) A prospective evaluation of cryopreservation strategiesin a two-embryo transfer programme. *Hum. Reprod*., **12,** 542–547.

Karlstro¨m, P.-O., Bergh,T., Forsberg, A.-S., Sandkvist, U. and Wikland, M. (1997) Prognostic factors for the success rate of embryo Freezing. *Hum. Reprod*., 12, 1263-1266.

Lane, M. and Gardner, D.K. (2005) Understanding cellular disruptions during early embryo development that perturb viability and fetal development. *Reprod. Fertil. Dev*., **17**, 371-378.

Leese, H.J., Sturmey, R.G., Baumann, C.G. and McEvoy, T.G. (2007) Embryo viability and metabolism: obeying the quiet rules. *Hum. Reprod.*,**22**, 3047-3050.

Leese, H.J., Baumann, C.J., Brison, D.R., McEvoy, T.G. and Sturmey, R.G. (2008) Metabolism of the viable mammalian embryo: quietness revisited. *Hum. Reprod.*,**14**, 667-672.

Mandelbaum, J., Junca, A.M., Plachot, M., Alnot, M.O., Alvarez, S., Debache, C., Salat-Baroux, J. and Cohen, J.

(1987) Human embryo cryopreservation, extrinsic and intrinsic parameters of success. *Hum. Reprod*., **2,** 709-715.

Mandelbaum, J., Belai'sch-Allart, J., Junca, A.-M., Antoine, J.-M., Plachot, M., Alvarez, S., Alnot, M.-O. and Salat-Baroux, J. (1998) Cryopreservation in human assisted reproduction is now routine for embryos but remains a research procedure for oocytes. *Hum. Reprod*., **13**, 161-174.

Nagy, Z., P., Taylor, T., Elliott, T., Massey, J.B., Kort, H.I. Shapiro, D.B. (2005) Removal of lysed blastomeres from frozen–thawed embryos improves implantation and pregnancy rates in frozen embryo transfer cycles. *Fertil. Steril*., **84**, 1606-1612.

Plachot, M., Belaisch-Allart, j., Mayenga, J.-M., Chouraqui, A., Tesquier, L. and Serkine, A.M. (2002) Outcome of conventional IVF and ICSI on sibling oocytes in mild male factor infertility. *Hum. Reprod*. **17**, 362-369.

Rienzi,L., Ubaldi, F., Iacobelli, M., Minasi, M.G., Romano, S., Ferrero, S., Sapienza, F., Baroni, E., Tesarik, J. and Greco, E. (2005) Developmental potential of fully intact and partially damaged cryopreserved embryos after laser-assisted removal of necrotic blastomeres and post-thaw culture selection. *Fertil. Steril*., **84**, 888-894.

Salumets, A., Tuuri, T., MaÈkinen, S., Vilska, S., Husu, L., Tainio, R. and Suikkari, A.-M. (2003) Effect of developmental stage of embryo at freezing on pregnancy outcome of frozen±thawed embryo transfer. *Hum. Reprod*., **18**, 1890-1895.

Salumets, A., Suikkari, A.-M., Mäkinen, S., Karro, H., Roos, A. and Tuuri, T. (2006) Frozen embryo transfers: implications of clinical and embryological factors on the pregnancy outcome. *Hum. Reprod*., **21**, 2368–2374.

Sifer, C., Sellami, A., Poncelet, C., Martin-Pont, B., Porcher, R., Hugues, J. and Wolf, J. (2006) Day 3 compared with day 2 cryopreservation does not affect embryo survival but improves the outcome of frozen-thawed embryo transfers. *Fertil. Steril.*, **86**, 1537-1540.

Stokes, P.J., Hawkhead, J.A., Fawthrop, R.K., Picton, H.M., Sharma, V., Leese, H.J. and Houghton, F.D. (2007) Metabolism of human embryos following cryopreservation: Implications for the safety and selection of embryos for transfer in clinical IVF Hum. *Hum. Reprod.*, **22,** 829-835.

Summers, M.C. and Biggers, J.D. (2003) Chemically defined media and the culture of mammalian preimplantation embryos: historical perspective and current issues. *Hum. Reprod*. *Up.*, **21**, 557-582.

Tang, R., Catt, J. and Howlett, D. (2006) Towards defining parameters for a successful single embryo transfer in frozen cycles. *Hum. Reprod*., **21,** 1179-1183.

Tiitinen, A., Halttunen, M., Härkki, P., Vuoristo, P. and Hyden-Granskog, C. (2001) Elective single embryo transfer: the value of cryopreservation. *Hum. Reprod*., **16**, 1140-1144.

Van den Abbeel, E., Camus, M., Van Waesberghe, L., Devroey, P. and Van Steirteghem, A.C. (1997) Viability of partially damaged human embryos after cryopreservation. *Hum. Reprod*., **12**, 2006-2010.

Van den Abbeel, E. and Van Steirteghem, A.C. (2000) Zona pellucida damage to human embryos after cryopreservation and the consequences for their blastomere survival and in vitro viability. *Hum. Reprod*., **15**, 373-378.

Van der Elst, J., Van den Abbeel, E.,Vitrier, S., Camus, M., Devroey, P. and Van Steirteghem, A.C. (1997) Selective transfer of cryopreserved human embryos with further cleavage after thawing increases delivery and implantation rates. *Hum. Reprod*., **12**, 1513-1521.

Ziebe, S., Bech, B., Petersen, K., Mikkelsen, A.L., Gabrielsen, A. and Andersen, A.N. (1998) Resumption of mitosis during post-thaw culture: a key parameter in selecting the right embryos for transfer. Hum. Reprod., **13**, 178–181.