EFFECTS OF EMBRYOLOGICAL PARAMETERS ON THE SUCCESS OF FRESH AND FROZEN EMBRYO TRANSFERS

ANDRES SALUMETS

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ANDRES SALUMETS

Department of Biosciences, Division of Animal Physiology
Faculty of Science, University of Helsinki, Finland
and
Infertility Clinic, The Family Federation of Finland
Helsinki, Finland

ACADEMIC DISSERTATION

To be publicly discussed, with the permission of the Faculty of Science, University of Helsinki, in the Lecture Hall 3, Biomedicum Helsinki, Haartmaninkatu 8, Helsinki, on October 3th, 2003, at 12 o'clock noon

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Supervised by

Docent Anne-Maria Suikkari, M.D., Ph.D.
Infertility Clinic, The Family Federation of Finland
Helsinki, Finland

Docent Timo Tuuri, Ph.D.
Infertility Clinic, The Family Federation of Finland
Helsinki, Finland

Reviewed by

Professor Outi Hovatta, M.D., Ph.D.
Department of Obstetrics and Gynaecology
Huddinge University Hospital, Karolinska Institute
Stockholm, Sweden

Professor Juha Tapanainen, M.D., Ph.D.
Department of Obstetrics and Gynaecology
Oulu University Hospital, University of Oulu
Oulu, Finland

Official opponent

Professor Arne Sunde, Ph.D.
Norwegian University of Science and Technology
Trondheim, Norway

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To my family

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LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original communications, which are referred to in the text by their Roman numerals:

I Salumets, A., Suikkari, A-M., Möls, T., Söderström-Anttila, V. and Tuuri, T. (2002). Influence of oocytes and spermatozoa on early embryonic development. *Fertility and Sterility*, 78, 1082–1087.

II Salumets, A., Hydén-Granskog, C., Suikkari, A-M., Tiitinen, A. and Tuuri, T. (2001). The predictive value of pronuclear morphology of zygotes in the assessment of human embryo quality. *Human Reproduction*, 16, 2177–2181.

III Salumets, A., Hydén-Granskog, C., Mäkinen, S., Suikkari, A-M., Tiitinen, A. and Tuuri, T. (2003). Early cleavage predicts the viability of human embryos in elective single embryo transfer procedures. *Human Reproduction*, 18, 821–825.

IV Salumets, A., Tuuri, T., Mäkinen, 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. *Human Reproduction*, 18, 1890–1895.

V Salumets, A., Horelli-Kuitunen, N., Suikkari, A-M., Metspalu, A. and Tuuri, T. Elevated incidence of chromosomally chaotic embryos among frozen-thawed preimplantation embryos. *European Journal of Obstetrics & Gynecology and Reproductive Biology, in press.*

ABBREVIATIONS

ANOVA	Analysis of variance
ATP	Adenosine tri-phosphate
COC	Cumulus-oocyte complex
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
EC	Early cleavage
eSET	Elective single embryo transfer
ET	Embryo transfer
FET	Frozen embryo transfer
FISH	Fluorescence *in situ* hybridisation
FSH	Follicle stimulating hormone
hCG	Human chorionic gonadotropin
ICSI	Intracytoplasmic sperm injection
IR	Implantation rate
IVF	*In vitro* fertilisation
MNB	Multinucleated blastomeres
NEC	No early cleavage
NPB	Nucleolar precursor body
OD	Ovum donation
OPU	Oocyte pick-up
PB	Polar body
PN	Pronucleus
PR	Pregnancy rate
PROH	1,2-propanediole
RNA	Ribonucleic acid
SET	Single embryo transfer
SD	Standard deviation
WHO	World Health Organisation
ZP	Zona pellucida

I. INTRODUCTION

Infertility is defined as the inability of a couple to become pregnant in a year without using any contraception (Barbieri, 1999). According to different estimates, 10–20% of couples are coping with involuntary childlessness (Barbieri, 1999). The birth of the first baby conceived *in vitro* opened up a completely new frontier in treatment of infertility (Steptoe and Edwards, 1978). Over the following 25 years, the pregnancy rates (PR) in *in vitro* fertilisation (IVF) procedures have constantly improved and nowadays the success rates of around 20–30% per cycle are routinely reported by majority of clinics (Nygren and Andersen, 2002). Unfortunately, the need to increase the PR has lead to transfer of multiple embryos resulting in unacceptably high (~30%) multiple pregnancy rate (Nygren and Andersen, 2002). The potential adverse effects of multiple pregnancies have stressed the need to limit the number of embryos transferred (Bergh et al., 1999). In order to achieve the total elimination of multiple pregnancies only single embryo should be transferred. Elective single embryo transfers (eSET) have steadily gained popularity and acceptance mostly in Scandinavian countries (Vilska et al., 1999; Martikainen et al., 2001; Tiitinen et al., 2001) and Belgium (Gerris et al., 1999) during the past few years. Performing eSET requires that in the selection of an embryo all parameters known to affect the viability of embryos should be carefully considered. Traditionally, the embryos are selected for transfer two days after insemination considering simultaneously their morphological appearance and cleavage rate (Puissant et al., 1987; Steer et al., 1992). Recently, two alternative strategies were implemented to find out the most viable embryos for transfer. Embryos could be chosen for transfer one day after insemination by evaluating either pronuclear (PN) stage embryo morphology (Scott and Smith, 1998; Tesarik and Greco, 1999) or early cleavage (EC) of zygotes (Shoukir et al., 1997; Sakkas et al., 1998). However, the importance of these novel approaches in eSET programmes has remained largely unexplored. The reduction of multiple pregnancies by increasingly applying eSETs requires also well-functioning embryo cryopreservation (Tiitinen et al., 2001). Therefore the increase in the proportion of eSET has paralleled the growth in number of frozen embryo transfers (FET). However, the use of FET results with nearly 40% fewer deliveries than fresh embryo transfer (ET) (STAKES, 2002). For the purpose of improving PR, all aspects of FET should be carefully evaluated.

II. REVIEW OF THE LITERATURE

1. Human conception

Most of the knowledge concerning human conception has been gathered from IVF of oocytes and subsequent embryo culture for alleviating infertility. IVF and embryo culture have made possible studies on the morphological aspects (Hardy et al., 1989) and the timing of early human embryo development (Trounson et al., 1982) as well as the genetic analysis of embryos (Plachot et al., 1987). The subsequent studies have also concentrated on the quality of gametes and the *in vitro* culture conditions as probably the most important factors affecting preimplantation embryo development.

1.1. Gametes and fertilisation

Genetically and functionally competent gametes are a prerequisite for normal fertilisation and early embryo development. Gametogenesis is the fundamental biological process by which haploid germ cells (oocytes and spermatozoa) are produced through meiosis. During meiotic divisions two rounds of chromosome segregation following a single round of DNA replication halve the diploid complement of chromosomes. Subsequent elaborate maturational processes prepare gametes for forthcoming developmental events. During fertilisation the haploid male and female gametes fuse, producing a diploid zygote that contains two copies of each chromosome.

1.1.1. Oocyte

The maturation of oocytes in IVF. During IVF the patients undergo pituitary suppression either with gonadotropin-releasing hormone agonists or antagonists. When suppression is achieved, maturation of a cohort of oocytes is accomplished with ovarian hyperstimulation using either urinary-derived gonadotropin injections, or more recently recombinant follicle stimulating hormone (FSH) preparations. During the follicular growth various ribonucleic acids (RNAs) and proteins accumulate in the cytoplasm of the oocyte (cytoplasmic maturation) (Gougeon, 1996). The final oocyte maturation is initiated by an injection of human chorionic gonadotropin (hCG), followed by transvaginal oocyte pick-up (OPU) 36 hours later. The primary oocyte undergoes the first meiotic division (nuclear maturation), resulting in the mature haploid oocyte and the first polar body (PB), between 15–28 hours post-hCG surge (Figure 1). The mature oocyte then promptly begins the second meiotic division but stops

soon thereafter at the second meiotic metaphase, which is completed after sperm entry and oocyte activation.

Several studies have demonstrated the relationships between follicular characteristics, fertilisation and embryo development after ovarian stimulation for IVF. Most of the studies have shown better fertilisation rates and embryo morphology for oocytes originating from follicles with a diameter ≥16 mm (Dubey et al., 1995; Ectors et al., 1997). Oocyte quality is also related to the concentrations of estardiol, progesterone and testosterone in the follicular fluid (Kreiner et al., 1987; Andersen, 1993). Measurements of perifollicular vascularity and blood flow by pulsed Doppler ultrasonography have suggested an additional link between intrafollicular conditions and oocyte competence. These studies have concluded that fully grown follicles exhibit unique blood flow characteristics, and that oocytes from well vascularized follicles are associated with a better IVF outcome (Van Blerkom et al., 1997; Bhal et al., 1999). Van Blerkom et al. (Van Blerkom et al., 1997) showed that follicles with poor perifollicular vascularisation had severely hypoxic conditions and oocytes derived from those follicles possessed numerous cytoplasmic defects and spindle anomalies. In addition, the embryos that developed from those oocytes often contained multinucleated blastomeres (MNB).

The morphological aspects of cumulus-oocyte complexes (COCs). Several layers of cells, called the cumulus oophorus, surround oocytes collected in IVF. Traditionally, the maturity of the oocytes is evaluated according to the appearance of the COCs. It has been suggested that an expanded cumulus indicates mature and good quality oocytes, while a compact cumulus characterizes immature oocytes (Veeck, 1999). However, there might be discrepancy between the nuclear maturity of the oocyte and the appearance of the COC (Laufer et al., 1984). Also, the mitotic activity of the cumulus-granulosa cells seems to be an important prognostic factor of oocyte competence, as a direct relationship has been found between an increased proliferating capacity of these cells and an elevated clinical PR (Gregory et al., 1994). The presence of apoptotic granulosa cells indicates low quality of the oocytes (Nakahara et al., 1997).

1.1.2. Spermatozoon

Spermatogenesis. Spermatogenesis is a complex process that can be divided into three major steps: (i) multiplication of spermatogonia by the process of mitosis; (ii) meiotic divisions whereby the chromosome number is reduced from diploid to haploid and (iii) transformation of the round spermatid into the complex structure of the spermatozoon. The resulting spermatozoon is released into the lumen of the seminiferous tubule and transported through the epididymis to the ejaculatory duct. During this passage the spermatozoa acquire both motility and fertilising ability.

Assessment of sperm quality. Traditionally the assessment of sperm quality is based upon the conventional semen profile, constructed according to recognised World Health Organisation (WHO) guidelines (WHO, 1999). Conventional semen analysis incorporates information about the volume of the ejaculate, the concentration, motility and morphology of the spermatozoa, and the presence of anti-sperm antibodies. A simple grading system consisting of four classes of spermatozoa has been devised to assess sperm motility (WHO, 1999). Within this classification system the motility of each spermatozoa is graded according to whether it shows rapid progressive motility (≥25 μm/s) (grade A), slow progressive motility (grade B), non-progressive motility (grade C) or immotility (grade D). Due to the variety of morphological malformations sperm morphology seems to be the least standardised parameter of semen analysis. Two most commonly used classification systems for evaluation of sperm morphology are WHO (WHO, 1999) and Tygerberg strict criteria (Kruger et al., 1986). Whilst conventional semen analysis maintains a central role in assessment of male fertility, a definitive decision of fertilising capacity of sperm often cannot be made as a result of a basic semen analysis. Therefore a series of different additional sperm functional tests have been developed, trying to get more precise diagnostic tools for sperm quality. These new tests have focused on the integrity of sperm chromatin, including both chromatin packaging anomalies and deoxyribonucleic acid (DNA) strand damage (Evenson et al., 1980).

1.1.3. Cellular processes occurring during fertilisation

Sperm-oocyte interaction and oocyte activation. The acrosome intact sperm attaches to the z*ona pellucida* (ZP) (Figure 1). The interaction of gametes induces a signal transduction cascade that culminates with acrosome reaction. During acrosome reaction the released hydrolytic enzymes digest a path through the ZP, along which the sperm passes into the perivitelline space. The subsequent fusion of gametes is followed by engulfment of the sperm by the oocyte cytoplasm, decondensation of the sperm nucleus, oocyte activation, cortical reaction (to avoid polyspermy) and completion of the second meiotic division. The extrusion of the second PB has been demonstrated to occur 2 hours after intracytoplasmic sperm injection (ICSI) (Nagy et al., 1994; Payne et al., 1997) and 3 hours after insemination (Lopata et al., 1980). The second PB is generally extruded immediately adjacent to the first PB. However, in 20% of all fertilised eggs the second PB is displaced >10° from the site of the first PB (Payne et al., 1997), although the degree of the angle between PBs is unrelated to subsequent embryo morphology (Garello et al., 1999).

The formation of pronuclei. The male and female PN (Figure 1), both of which contain haploid genomes, are usually formed simultaneously and can be visualised using light microscopy as early as 3 hours post-injection (Payne et

al., 1997) and 5–6 hours after insemination (Veeck, 1999). Asynchronous appearance of PN, occurring in only a small fraction of fertilised eggs (13%), has been found to be associated with poor embryo morphology (Payne et al., 1997). Pronuclear development occurs approximately 4 hours sooner in ICSI than in IVF, as all fertilised oocytes had two pronuclei 10 hours after injection but not sooner than 14 hours after insemination (Nagy et al., 1998).

The male PN appears near the site of sperm entry whereas the female PN forms close to the second PB (Payne et al., 1997). The sperm centrosome forms the microtubular organising centre that brings the PN together and pushes them towards the oocyte centre. Defects in this dynamic process may lead to disorders of fertilisation and early embryonic development (Asch et al., 1995). The male and female PN enlarge during the final stages of pronuclear movement and attain final diameters of 24 μm and 22 μm, respectively (Payne et al., 1997). Significant PN size asynchrony has been related to oocyte postmaturity (Goud et al., 1999) and chromosomal abnormalities (Table I) (Sadowy et al., 1998; Manor et al., 1999). Pronuclei may rotate within the ooplasm and direct their axis towards the second PB, probably in preparation for subsequent cleavage. Zygotes that failed to achieve an optimal PN orientation exhibit extensive fragmentation and uneven blastomere cleavage during further development (Garello et al., 1999). The replication of DNA starts synchronously within both PN at around 9–10 hours after insemination and is completed approximately 3–5 hours later (Balakier et al., 1993). Although the major activation of the human embryonic genome occurs at the 4–8-cell stage (Braude et al., 1988), a minor transcriptional activity has been detected also in zygotes (Ao et al., 1994).

Cytoplasmic halo. The withdrawal of mitochondria from the cortex of the human zygote (i.e. the formation of a cytoplasmic halo) and the subsequent accumulation around the opposed PN can be visually followed during the pronuclear formation (Figure 1) (Payne et al., 1997). It has been argued that one reason for mitochondrial accumulation around the PN could be the elevated energy demand of developing pronuclei (Bavister and Squirrel, 2000). It seems that this process might represent an important step in early embryogenesis as it is associated with an elevated blastocyst formation rate (Zollner et al., 2002). The appearance of the cytoplasmic halo has been shown to vary from relatively symmetrical to grossly asymmetrical (Van Blerkom et al., 2000). Zygotes with an asymmetrical halo developed into embryos showing reduced mitochondrial inheritance and diminished ATP generating capacity in some of the blastomeres. These blastomeres often remained undivided and frequently died during subsequent culture.

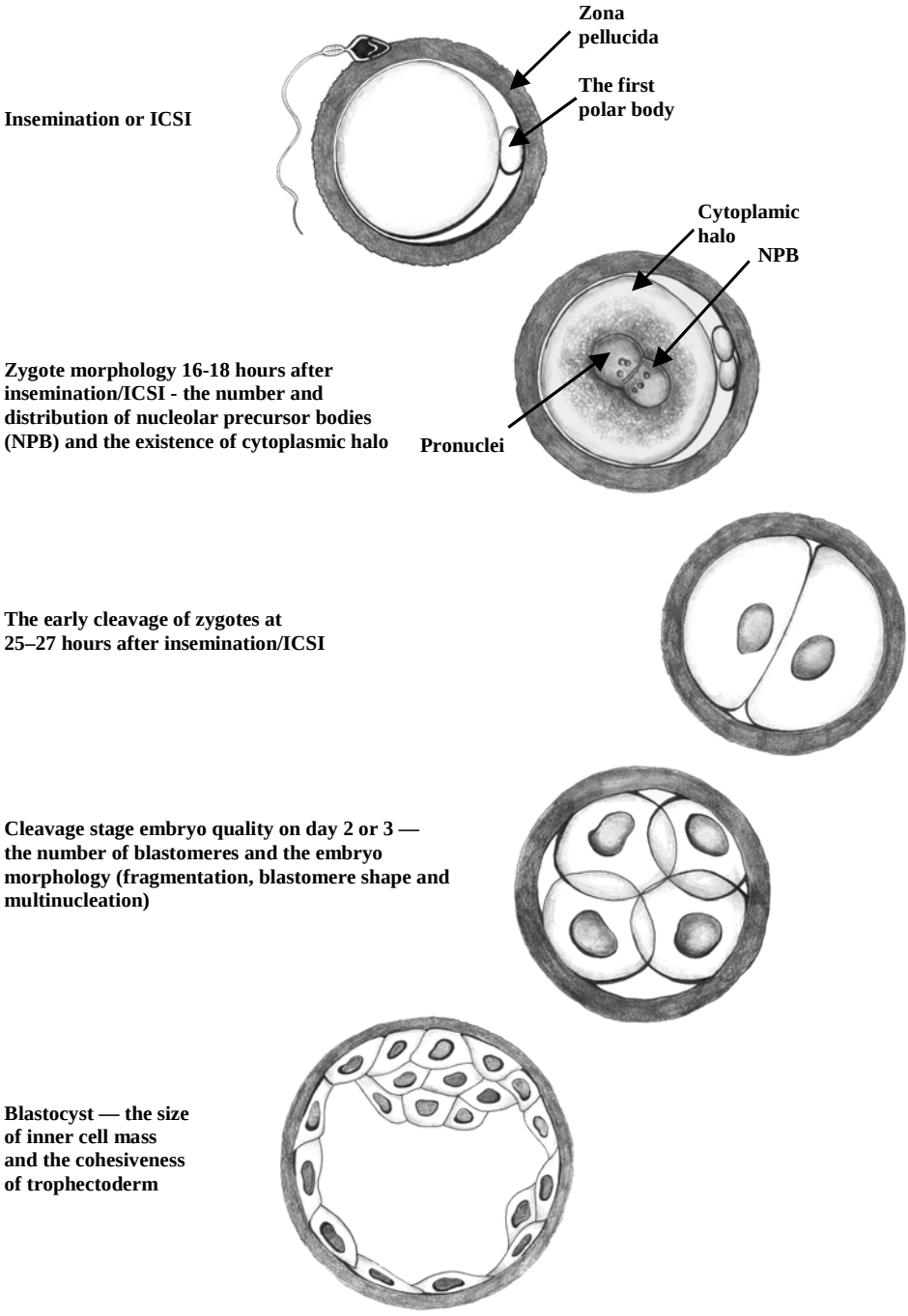

Figure 1. Preimplantation embryo development and important aspects of embryo selection for transfer

Nucleolar precursor bodies. During PN formation nucleoli can be seen within both PN (Wright et al., 1990). The number of nucleoli within PNs varies from one to roughly ten and characteristically fewer nucleoli are seen in female (4) than in male (7) PN (Payne et al., 1997). The nucleoli in human zygotes are thought to be inactive in the sense of ribosomal RNA synthesis and therefore these structures are called nucleolar precursor bodies (NPB) (Figure 1). It has been shown that during PN development NPB may accumulate in the contact area of pronuclei (Wright et al., 1990). In some studies the alignment of NPB has been considered as a sign of developmental competence because these zygotes yielded significantly better quality embryos on day 3 (Tesarik and Greco, 1999) and demonstrated an elevated blastocyst formation rate (Figure 2) (Scott et al., 2000). The sperm cell seems to have an effect on the localisation and number of NPB, as was shown in a recent study exploiting donor oocyte-sharing programme (Tesarik et al., 2002). In that study, the zygote stage morphology was compared between two recipient couples of the same oocyte donor and a clear difference in the proportion of zygotes with abnormal PN morphology was found. Although only limited information is available concerning why chromatin and NPB polarise in human zygotes, some authors believe that this process might reflect an early step in the formation of embryonic axes that can regulate human preimplantation embryo development (Scott, 2000).

1.1.4. Fertilisation in IVF and ICSI procedures

Fertilisation in IVF. In conventional IVF oocytes are fertilised either in test tubes or culture dishes using ~25 000 motile spermatozoa per oocyte. The gametes are co-incubated for 16–18 hours, after which the fertilisation of oocytes (i.e. the presence of two PN and PB) is examined. The long exposure of oocytes to spermatozoa has been shown to reduce the fertilisation rate and embryo quality, probably because of the reactive oxygen species produced by both normal and abnormal spermatozoa as well as by activated leucocytes (Aitken, 1994; Gianaroli et al., 1996a; Gianaroli et al., 1996b). Improved results in terms of fertilisation and embryo development have been achieved by shortening the insemination time to 1 hour (Gianaroli et al., 1996a; Gianaroli et al., 1996b).

Fertilisation rates of about 60–70% are routinely reported by a majority of IVF programmes. Numerous studies have examined the factors influencing the efficiency of fertilisation in normal IVF procedures. All three basic sperm parameters (concentration, motility and morphology) have been found to affect the fertilisation of oocytes. A correlation has been established between sperm concentrations in native ejaculates and the success of fertilisation (Biljan et al., 1994). Low fertilisation rate has also been demonstrated in patients with impaired sperm motility, with a cut-off value of 30% for progressive motility

(Enginsu et al., 1992). However, the best predictor of the fertilisation potential of semen seems to be the sperm morphology. Several studies have indicated that sperm morphology as evaluated by either WHO (Duncan et al., 1993) or Tygerberg strict criteria (Kruger et al., 1986; Kruger et al., 1988) is closely related to fertilisation rate. Hinting et al. have suggested a threshold value of 16% for normal sperm morphology based on WHO criteria (Hinting et al., 1990). In a meta-analysis by Coetzee et al. patients having <4% normal spermatozoa, according to Tygerberg strict criteria, had a fertilisation rate of 59.3%, whereas those who had >4% normal spermatozoa had a fertilisation rate of 77.6% (Coetzee et al., 1998). In addition, a negative correlation has been found between the percentage of sperm cells with fragmented DNA and the fertilisation rate (Sun et al., 1997).

Fertilisation in ICSI. In ICSI a single sperm is chosen and injected directly into the cytoplasm of the oocyte, thus bypassing several important steps involved in sperm-egg recognition and gamete fusion (Palermo et al., 1992). ICSI was initially used for cases in which fertilisation of oocytes was not achieved by conventional IVF, especially because of a low sperm count, poor morphology and low motility. Subsequently spermatozoa extracted from the epididymis and testis have also been used as a source of male gametes in azoospermic patients (Silber et al., 1994). Fertilisation rates of around 60–70% can be obtained with ICSI, and the efficiency of this procedure is unrelated to any of the standard semen characteristics (Svalander et al., 1996). Normal fertilisation and embryo development have been achieved even with acrosomeless (“round-headed”) (Lundin et al., 1994) and immotile spermatozoa (Stalf et al., 1995). The only factor that appears to influence fertilisation in ICSI is sperm DNA strand integrity (Lopes et al., 1998).

Denudation of oocytes prior to an ICSI procedure has revealed that a significant proportion of oocytes exhibit different morphological abnormalities, such as excessive granularity, vacuolarisation, clustering of smooth endoplasmatic reticulum, refractile bodies, large perivitelline space and fragmented PB. The extent to which these abnormalities can interfere with the normal process of fertilisation following ICSI is controversial. Results of a study by Xia et al. have suggested that oocyte morphology is significantly related to the fertilisation rate after ICSI (Xia, 1997). In other studies, however, no correlation between oocyte morphology and the fertilisation rate have been demonstrated (De Sutter et al., 1996; Serhal et al., 1997; Balaban et al., 1998; Kahraman et al., 2000; Meriano et al., 2001).

1.2. Human preimplantation embryo development

Following fertilisation the zygote begins to divide, forms the morula and thereafter progresses to the blastocyst stage (Figure 1). By day 4 the blastocyst

reaches the uterus, expands and hatches from the ZP. The blastocyst attaches to the uterine wall between day 7–9 and embeds itself in the endometrium. In IVF, the embryos are transferred to the uterus usually on days 2–3 or day 5. The embryo quality has been shown to be comparable in IVF and ICSI procedures (Palermo et al., 1996; Staessen et al., 1999; Verheyen et al., 1999).

1.2.1. Zygote cleavage

Several authors have studied the timing of PN breakdown and cleavage of zygotes at normal IVF (Trounson et al., 1982; Balakier et al., 1993; Capmany et al., 1996) and ICSI (Nagy et al., 1994). The progression of the human zygote to the two-cell stage can occur as soon as 20 hours after insemination, although the majority of zygotes start dividing 25–27 hours post-insemination (Balakier et al., 1993). Before the first cleavage the sperm centrosome divides and forms the two centers of the first division spindle. In humans the PN do not fuse, and the combination of parental genomes (syngamy) occurs only after the pronuclear breakdown when maternal and paternal chromosomes intermingle and align on the metaphase plate of the first division. Early cleavage of zygotes to the two-cell stage by 25–27 hours post-insemination or ICSI has been reported to be a clinically relevant sign of embryo competence (Shoukir et al., 1997; Sakkas et al., 1998). It has been demonstrated that EC embryos have better morphology on day 2 (Lundin et al., 2001) and higher blastocyst formation rate (Fenwick et al., 2002) than non-early cleavage (NEC) embryos. In the study by Lundin et al. 22% and 35% of IVF and ICSI zygotes, respectively, possessed two cells at 25–27 hours after insemination or ICSI (Lundin et al., 2001). The reasons why EC embryos have higher competence remain largely obscure, although the answer may lay in the quality of the oocytes (Lundin et al., 2001). Semen characteristics have not been shown to have any effect on the timing of the first cleavage of either IVF (Shoukir et al., 1997) or ICSI (Sakkas et al., 1998) zygotes.

1.2.2. Embryo cleavage

Trounson et al. have studied the timing of the human preimplantation embryo development *in vitro*, and the mean times for the first three blastomere cleavages were 35.6, 45.7, 54.3 hours after insemination (Trounson et al., 1982). A significant number of human embryos have been shown to arrest between the 4–8-cell stages (Bolton et al., 1989). Gene expression in human embryos first occurs at the same developmental stage and failure of embryonic genome activation has been proposed as one possible reason for cleavage arrest (Braude et al., 1988). Human embryos *in vitro* possess different numbers of blastomeres at the second and third day of development, probably reflecting the varieties in cleavage rates. The differences in cleavage rates may be related to

the quality of gametes. The significance of the oocyte quality on embryo cleavage rate may be due to an intensive accumulation of proteins and RNAs in the cytoplasm of the oocytes during their maturation (Gougeon, 1996). This endowment of molecules is essential for normal embryo development in the course of the first two or three days while the embryonic genome is silent. There might also be the effect of sperm cell on the blastomeres cleavage rate (Palermo et al., 1994). The most important cellular contribution of the sperm cell to the embryo is centrosome, an organelle that regulates cell divisions during early embryonic development. In the study by Ron-El et al. delayed fertilisation and subsequent development were found to be associated with impaired sperm morphology (Ron-El et al., 1991). In addition, the blastomere cleavage rate might have a genetic basis as in mice Ped (preimplantation embryo development) gene regulating embryo growth rate has been identified (Warner et al., 1998). So far, all attempts to find the human homologue of this gene have failed.

1.2.3. Cleavage stage embryo morphology

A feature of human preimplantation embryo development *in vitro* is the high prevalence of morphological abnormalities, including uneven cleavage, blastomere fragmentation, multinucleation and *zona pellucida* anomalies. The precise cleavage of zygotes and blastomeres into two equally sized daughter cells relies upon the position of the spindle and the functional activity of cytoskeletal elements. Slight variations in blastomere sizes within the same embryo are probably unimportant, but major differences may indicate defects in underlying cellular processes (Hardarson et al., 2001).

Blastomere fragmentation. Blastomere fragmentation is a morphological abnormality observed in ~40% of the human early embryos (Antczak and Van Blerkom, 1999). In these embryos the fragments first appeared at 1-cell (25%), 2-cell (40%) and ≥4-cell (35%) stages. Several hypotheses have been put forward to explain the biological mechanisms behind this phenomenon, including: (i) the insufficient adenosine tri-phosphate (ATP) production (Van Blerkom et al., 2001); (ii) apoptotic processes (Jurisicova et al., 1996) and (iii) poor quality of either oocytes (Xia, 1997) or sperm cells (Parinaud et al., 1993). A significant amount of data has been collected concerning the detrimental effect of fragmentation on the embryo development. The study by Bolton et al. was the first to report that human embryos exhibiting considerable extracellular fragmentation are less able to reach blastocyst stage than good quality embryos (Bolton et al., 1989). In the study of Alikani et al., the relationship between the degree of fragmentation and the incidence of blastulation were examined (Alikani et al., 2000). The blastocyst formation rate was significantly higher

among embryos with 0–15% fragmentation (33%) than among embryos with >15% fragmentation (17%).

Multinucleated blastomeres. Although the majority of the embryos contain a single nucleus in each blastomere, some of the embryos may however possess blastomeres with more than one nucleus. Initially, these so-called multinucleated blastomeres were associated with arrested embryos, but subsequently they were also identified in normally developing embryos. It has been observed that 17% of embryos at the 2–4-cell stage had at least one MNB, and the proportion increased to 65% at the 9–16-cell stage (Hardy et al., 1993). Comparing the volumes of multi- and mononucleated blastomeres it was revealed that MNB arise from random failures of cytokinesis (Hardy et al., 1993). Other possible mechanisms involved in the formation of MNB include the partial fragmentation of nuclei and defective migration of chromosomes at mitotic anaphase (Winston et al., 1993). Despite extensive investigations the scientists still do not completely understand the pathological mechanisms triggering the formation of MNB. It has been argued that MNB could be related to sub-optimal culture conditions (Pickering et al., 1995); adverse effects of cooling on cytoskeleton (Pickering et al., 1990); hypoxic intrafollicular conditions (Van Blerkom et al., 1997) or accelerated ovulation induction (Jackson et al., 1998).

1.2.4. Morula and blastocyst

By the fourth day of development the human preimplantation embryo contains approximately 16 blastomeres and is called morula. The formation of blastocyst is initiated between the fourth and fifth day of development when embryo contains ~32 blastomeres (Hardy et al., 1989). The two cell types of blastocyst, namely the inner cell mass and trophectoderm cells develop from approximately 12 and 20 cells located, respectively, at the centre and outside of the morula. The inner cell mass gives rise to all the tissues of the fetus, while trophectoderm forms a fluid-transporting epithelium responsible for blastocyst expansion and subsequently establishes the placenta. The use of sequential culture media that have been specially designed for the changing requirements of the embryos allows approximately half of all zygotes to develop to blastocyst stage (Gardner et al., 1998). However, fewer cells have been observed in *in vitro* blastocysts than in blastocysts obtained after uterine flushing (>150 cells) (Croxatto et al., 1972). Significant correlations have been found between the number of blastocysts and the numbers of oocytes, zygotes and eight-cell embryos (Jones et al., 1998; Shapiro et al., 2000). Embryos with good morphology have better chance to reach the blastocyst stage than other embryos (Bolton et al., 1989; Alikani et al., 2000). However, the predictive value of embryo morphology on day 2 or 3 for subsequent blastocyst formation seems to be rather limited

(Rijnders and Jansen, 1998; Graham et al., 2000). In addition, a paternal influence on blastocyst formation rate has been suggested as impaired blastocyst formation has been observed in conjunction with poor sperm quality (Janny and Meneso, 1994; Shoukir et al., 1998).

1.3. Genetic aspects of preimplantation embryos

The results of a number of fluorescence *in situ* hybridisation (FISH) studies have been uniform in the view that significant numbers (30–60%) of preimplantation embryos possess chromosomal abnormalities, and this may contribute to the low PR after IVF. There is an agreement that embryos with good morphology contain less frequently chromosomal aberrations than arrested or poor-morphology embryos (Table I). Also, correlations between some distinct morphological abnormalities of embryos and an increased level of chromosomal aberrations are well established (Table I).

Table I. Different categories of human preimplantation embryos and chromosomal abnormalities

Embryo category	**Chromosomes studied**	**Results**	**Reference**
Normally developing IVF and ICSI embryos			
IVF embryos	13,18,21,X,Y	43% normal	(Munne et al., 1995)
IVF embryos	1,17 or X,Y	70% normal	(Harper et al., 1995)
IVF embryos	1,4,6,7,14,15,17,18,22	34% normal	(Bahce et al., 1999)
IVF vs. ICSI embryos	13,18,21,X,Y	39 vs. 48% normal	(Munne et al., 1998)
Cryopreserved embryos			
Arrested embryos	1,X,Y	20% normal	(Laverge et al., 1998)
Arrested and cleaved embryos	15,16,17,18,X,Y	25% normal	(Iwarsson et al., 1999)
Zygotes and embryos with morphological abnormalities			
Zygotes with uneven PN	13,18,21,X,Y	24% normal	(Manor et al., 1999)
Embryos with MNB	13,18,21,X,Y	23% normal	(Kligman et al., 1996)
Fragments 0–15% 20–40% 45–100%	13,18,21,X,Y	60% normal 45% normal 0% normal	(Munne and Cohen, 1998)
Embryos with dominant single blastomere	18,X,Y	mostly polyploid	(Munne et al., 1994)

Magli et al. have shown that chromosomally abnormal embryos are able to reach the blastocyst stage, but more often their development is arrested (Magli et al., 2000). A number of different chromosomal abnormalities (aneuploidy, mosaicism, polyploidy and haploidy) have been found in early embryos. Aneuploidy and mosaicism are, respectively, the most prevalent types of abnormalities in morphologically normal and abnormal embryos (Munne and Cohen, 1998). It has been demonstrated that in human embryos the incidence of aneuploidy increases with maternal age, thereby corroborating the fact that women of advanced reproductive ages produce substantially more frequently oocytes with chromosomal abnormalities (Munne et al., 1995; Dailey et al., 1996). As the chromosomal aberrations of mosaic and chaotic embryos arise during blastomere divisions, their prevalences have been shown to increase during embryo development (Bielanska et al., 2002).

2. Factors affecting the success of fresh embryo transfer

The average PR reported by majority of IVF clinics range around 20–30% per cycle, being comparable in IVF and ICSI procedures (Nygren and Andersen, 2002). The PR per cycle has improved from 21.4% in 1992 to 25.2% in 2000 in Finnish IVF clinics (STAKES, 2002). During the same time the delivery rate has increased from 16.5% to 19.6% (STAKES, 2002). The success of fresh embryo transfer is determined by two factors: embryo quality and uterine receptivity. Routinely the best embryo(s) is selected for transfer 2–3 days after insemination or ICSI considering simultaneously their morphological appearance and cleavage rate (Puissant et al., 1987; Steer et al., 1992). More promising future strategies for embryo selection can be based on the assessment of embryo metabolism (Houghton et al., 2002) and/or preimplantation genetic diagnosis (Gianaroli et al., 1997; Munne et al., 1999). According to some studies, the blastocyst culture and transfer could also be successfully applied as a mean to improve the success of fresh embryo transfer (Gardner et al., 1998; Marek et al., 1999).

2.1. The relationship between embryo morphology and viability

2.1.1. Zygote morphology and embryo viability

During the last years some evidence has been gathered indicating that the developmental potential of human embryos may already be evaluated at the zygote stage (Scott and Smith, 1998; Tesarik and Greco, 1999). The zygote scoring systems comprise morphological aspects and localisation of pronuclei as well as cytoplasmic appearance. Selection of embryos for transfer at the

zygote stage would be the most beneficial in countries with a strict embryo protection law, such as Germany and Switzerland. In these countries the law prohibits the selection of cleavage stage embryos, and therefore only the embryos that are planned to be transferred are left in culture (Montag and van Der Ven, 2001). Zygote scoring systems would offer the possibility to culture and transfer only the embryos with the highest chance of implantation.

The zygote scoring system by Scott and Smith. Scott and Smith (1998) reported the use of a zygote scoring system in embryo selection for the first time (Figure 2) (Scott and Smith, 1998). The authors used an empirically derived zygote evaluation system, which considered the position of NPB, existence of a cytoplasmic halo and progression to the 2-cell stage by 24–26 hours after insemination (Figure 1). The retrospective analysis revealed that the implantation rate (IR) of halo-positive zygotes with polarised distribution of NPB and progression to the 2-cell stage by 24–26 hours post-insemination was higher (28%) than the IR of embryos with a scattered distribution of NPB and homogenous cytoplasm (2%) (Scott and Smith, 1998). The same group has collected further evidence to support their initial conclusions (Scott et al., 2000).

The zygote scoring system by Tesarik and Greco. Another zygote scoring system was proposed by Tesarik and Greco (Figure 2) (Tesarik and Greco, 1999). In this system zygotes were divided, based on the number and distribution of NPB, between one normal (pattern 0) and five abnormal classes (patterns 1–5). The zygotes in the normal class (pattern 0) had all NPB in both PN either polarised or unpolarised, emphasising the importance of synchrony between the pronuclei. A substantially higher clinical PR was demonstrated after transfer of at least one embryo having the normal PN pattern (50%) than after the transfer of only abnormal embryos (9%) (Tesarik and Greco, 1999). Subsequent studies by the same group (Tesarik et al., 2000) and others (Wittemer et al., 2000) have also corroborated the usefulness of Tesarik's zygote classification system. Recently, a slightly modified Tesarik's zygote classification system was introduced (Montag and van Der Ven, 2001). The initial pattern 0 was subdivided into two new patterns, which were specified as 0A (7>NPB, equally distributed) and 0B (7≤ NPB, polarised). In this study the cycles with transfer of at least one embryo derived from pattern 0B zygotes resulted in significantly higher rates of pregnancy (37.9%) and implantation (20.5%) than all other ETs (26.4% and 15.7%).

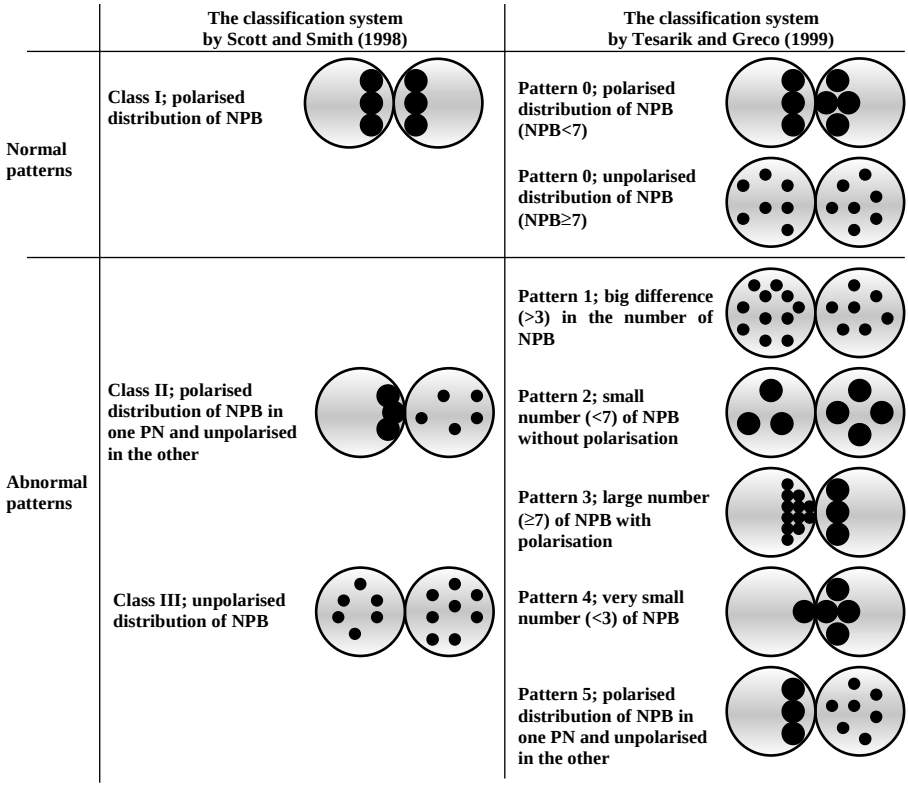

Figure 2. Two different classification systems for evaluation of pronuclear morphology

2.1.2. The effect of early cleavage stage embryo morphology on the pregnancy rate

Blastomere fragmentation. Estimation of the degree and pattern of blastomere fragmentation is included in most embryo evaluation schemes. It has been generally accepted that there is an inverse relationship between the degree of extracellular fragmentation and the implantation potential of embryos (Hill et al., 1989; Erenus et al., 1991; Scott et al., 1991; Staessen et al., 1992; Giorgetti et al., 1995; Ziebe et al., 1997; Alikani et al., 1999). In the study of Staessen et al. embryos were divided into three classes according to the degree of fragmentation: type A (no fragments), type B (<20% fragmentation) and type C (>20% fragmentation) (Staessen et al., 1992). It was shown that both types A and B had a similarly high IR when compared to type C embryos. In another study the results of 957 single embryo transfers (SET) were evaluated (Giorgetti et al., 1995). The analysis revealed that the presence of anucleate fragments was

associated with significantly lower IR (8.1% versus 11.5%) (Giorgetti et al., 1995). Ziebe et al. analysed 1001 IVF cycles where embryos with identical morphological appearance and cleavage stage were transferred (Ziebe et al., 1997). In that study, similar IR were found for embryos without fragments and <10% fragments. However, these embryos had a significantly higher chance of implantation (21%) than embryos with >10% fragmentation (5%).

Alikani and Cohen (1999) provided a more elaborate classification system for embryo fragmentation considering both the degree and pattern of fragmentation (Alikani et al., 1999). However, in that study, assisted hatching and fragment removal were performed for those embryos possessing >5% fragmentation. Contrary to the study of Ziebe et al. (Ziebe et al., 1997) in which the decreased implantation of embryos was observed when the fragmentation exceeded 10% of the perivitelline space, Alikani et al. found that embryos having up to 35% fragmentation implanted with comparable success (Alikani et al., 1999). In addition, a correlation was found between the pattern of embryo fragmentation and the capacity for implantation. Embryos having large fragments had a markedly lower implantation potential (18%) than other embryos (32%).

Blastomere shape. The view that human embryos with unevenly sized blastomeres have lower implantation potential than evenly cleaved embryos stems largely from three studies (Giorgetti et al., 1995; Ziebe et al., 1997; Hardarson et al., 2001). By analysing SET, Giorgetti et al. showed the importance of equal blastomere divisions, as embryos with irregular cells were almost two times less likely (6.9%) to implant than embryos with symmetrical cells (11.7%) (Giorgetti et al., 1995). Also in another study the embryos containing blastomeres of irregular size had lower IR compared to embryos having uniformly sized blastomeres (Ziebe et al., 1997). An article recently published by a Swedish group agreed with previous studies and showed significantly impaired IR for unevenly cleaved embryos (23.9%) when compared with evenly cleaved embryos (36.4%) (Hardarson et al., 2001).

Embryos with MNB. Although embryos with MNB are able to implant the PR are expected to be low (Balakier and Cadesky, 1997). After transfers of only multinucleated embryos or multinucleated and mononucleated embryos together, the clinical PR per transfer (13.2%) was lower when compared to transfers of solely mononucleated embryos (23.2%) (Pelinck et al., 1998). It has also been noted that when >50% of transferred embryos contained MNB there was a reduction in implantation (3.4% vs. 14.7%) and clinical pregnancy (9.1% vs. 29.1%) rates, when compared with transfers of control embryos (Jackson et al., 1998).

2.2. Embryo cleavage rate as a predictor of the developmental potential of embryos

2.2.1. EC of zygotes

The early cleavage of zygotes that occurs by 25–27 hours after fertilisation has been found to be a reliable indicator of embryo viability both in normal IVF (Shoukir et al., 1997) and ICSI (Sakkas et al., 1998). The clinical PR of IVF patients in EC embryo transfer group was 33%, while only 15% of patients in NEC embryo transfer group become pregnant (Shoukir et al., 1997). There was an even bigger difference between EC and NEC embryo transfers in ICSI, as patients who received EC embryos exhibited a 4-fold higher clinical PR (26%) than those who had NEC embryos transferred (6%) (Sakkas et al., 1998). The trend that EC embryos yield more pregnancies than NEC embryos has also been observed in other studies (Bos-Mikich et al., 2001; Lundin et al., 2001; Petersen et al., 2001; Sakkas et al., 2001). When transferring up to six embryos more pregnancies occurred in the EC (55%) than in NEC embryo transfer group (25%) (Bos-Mikich et al., 2001). Similarly, in the study by Sakkas et al. the patients who received EC embryos had a higher clinical PR (45%) than other patients (24%) (Sakkas et al., 2001). In that study, markedly improved results in terms of successful implantations were achieved in weeks during which the embryo selection was based on the timing of the first cleavage. In the most comprehensive study to date, the transfers of EC embryos resulted with higher birth rate (34%) than transfers of NEC embryos (24%) (Lundin et al., 2001). Furthermore, the spontaneous abortion rate was significantly reduced in the EC (12.1%) compared to the NEC (20.2%) ET group.

2.2.2. Embryo cleavage rate and outcome of IVF procedure

The estimation of embryo cleavage rate is usually accomplished by determining the blastomere number 2–3 days after OPU. Embryos possessing more blastomeres are considered to cleave faster than their counterparts having lower numbers of cells. There is substantial clinical evidence suggesting that embryos with a slower cleavage rate *in vitro* are less likely to produce pregnancies following IVF-ET (Cummins et al., 1986; Claman et al., 1987; Staessen et al., 1992; Giorgetti et al., 1995; Ziebe et al., 1997). Cummins et al. were the first to indicate the positive relationship between embryo cleavage rate and implantation (Cummins et al., 1986). It was subsequently shown that PR was higher in a group of patients receiving at least one 4-cell embryo (26%) than patients receiving embryos developing at a slower rate (7%) (Claman et al., 1987). Ziebe et al. (1997) demonstrated that the transfer of 4-cell embryos even in the presence of minor fragmentation should be preferred to the transfer of 2-cell embryos without fragmentation (Ziebe et al., 1997). In that study the

implantation potential of 2-cell embryos without any fragmentation was substantially lower (13%) when compared to 4-cell embryos with <10% (32%) or without (23%) fragmentation. The analysis of SETs has also demonstrated that embryo cleavage rate has a profound effect on the outcome of IVF (Giorgetti et al., 1995; Vilska et al., 1999).

2.3. Developmental stage of transferred embryos and pregnancy rate of IVF

A large number of *in vitro* human embryos have been shown to arrest between the second and third day of development, probably because they are unable to activate transcription (Bolton et al., 1989). Therefore, it has been suggested that ET should be performed on day 3 after OPU in order to exclude the transfer of embryos that may stop dividing during that sensitive period. Several studies have investigated the effect of delaying ET to the third day post-insemination, but with controversial results. Some studies have demonstrated that performing ET on day 3 instead of day 2 improved the PR of IVF (Dawson et al., 1995; Carrillo et al., 1998), while others have found no difference between day 2 and day 3 transfers (Huisman et al., 1994; Ertzeid et al., 1999; Laverge et al., 2001). The postponing of ET until embryos have reached the blastocyst stage has also been suggested as a possible way to select the most viable embryos for transfer. A study performed by Gardner and his co-workers (1998) demonstrated the superiority of blastocyst transfers over early cleavage stage ETs (Gardner et al., 1998). The IR after blastocyst transfer (50.5%) was higher compared to the early cleavage stage ET (30.1%). The same conclusion was reached in other studies, where the using of blastocyst transfers was more successful than early cleavage stage ETs (Milki et al., 2000; Van Der Auwera et al., 2002). However, in other studies prolonging embryo culture from 2–3 to 5 days did not improve the PR of IVF (Scholtes and Zeilmaker, 1996; Coskun et al., 2000; Huisman et al., 2000).

2.4. Clinical factors determining the success rate of IVF

It is well accepted that the outcome of an IVF is affected not only by embryo quality, but also by clinical factors. Several studies have shown that patient age is one of the most important factors determining the PR in IVF (Piette et al., 1990; Roseboom et al., 1995). Impaired developmental competence of embryos from older women is likely caused by an increased incidence of chromosomal abnormalities in oocytes (Munne et al., 1995). Many reports suggest a relationship between aetiology of infertility and outcome of IVF. The negative impact of hydrosalpinx on the probability of pregnancy in IVF is firmly established (Blazar et al., 1997). Lower PR (Cano et al., 1997) and a higher abortion rate

(Ludwig et al., 1999) have been found in conjunction with polycystic ovarian syndrome. Endometriomas are also known to adversely effect the PR following IVF treatment (Yanushpolsky et al., 1998). The type of ovulation induction protocol used in IVF seems to have a profound effect on the success of the procedure. The use of gonadotropin-releasing hormone agonists in ovarian stimulation has been shown to result in increased numbers of collected oocytes and thereby improved PR (Liu et al., 1992). Gonadotropin-releasing hormone antagonists have recently been introduced into clinical practice and their safety and effectiveness have been confirmed (Albano et al., 2000). The application of recombinant FSH also appears to be more effective than urinary FSH (Bergh et al., 1997).

The number of transferred embryos. IVF usually involves the transfer of multiple embryos to improve the chance of success. However, the elevated PR parallels the increased occurrence of multiple pregnancies. It has been shown that a high frequency (~30%) of multiple pregnancies and births is the main factor that leads to adverse outcomes of IVF (preterm births, low birthweight and malformations) (Bergh et al., 1999). Templeton and Morris have established that the overall PR is not diminished, but the multiple PR is reduced after transferring two instead of three or more embryos (Templeton and Morris, 1998). The sole strategy to totally avoid multiple pregnancies is to transfer only single embryo (Gerris et al., 1999; Vilska et al., 1999; Martikainen et al., 2001; Tiitinen et al., 2001). In the study by Vilska et al., the PR (29.7%) after eSETs was comparable to the PR after two-embryo transfers (29.4%) (Vilska et al., 1999).

3. Factors influencing the success of FET

Embryo cryopreservation is an essential part of IVF, allowing all good quality spare embryos to be stored for later use (Zeilmaker et al., 1984). Embryo cryopreservation offers several important benefits: it provides the mean to reduce the number of transferred embryos in fresh and frozen ETs, thereby diminishing the risk of multiple pregnancies (Martikainen et al., 2001; Schnorr et al., 2001; Tiitinen et al., 2001); it allows to maximise the cumulative PR (Bergh et al., 1995) and finally it makes possible to cancel ET if a woman has the risk of ovarian hyperstimulation syndrome (Tiitinen et al., 1995). The PR after FET is around 15% per transfer but remain lower than that reported for fresh ET (Nygren and Andersen, 2002). This could be explained by damages of embryos caused by freezing and thawing procedures. Loss of blastomeres is one of the most deleterious effect of cryopreservation (Edgar et al., 2000). In addition, damages of cell membranes (Ng et al., 1988) and ZP (Cohen et al., 1988) have also been identified in thawed embryos. The developmental capacity

of frozen-thawed embryos can be further impaired by chromosomal defects possible induced by cryopreservation procedure (Laverge et al., 1998; Iwarsson et al., 1999). Other factors influencing the success of FET include the aetiology of infertility (Wang et al., 2001), age of the women (Schalkoff et al., 1993; Wang et al., 2001), the type of ovarian stimulation protocol used before OPU (Van der Elst et al., 1996), outcome of the fresh ET (Lin et al., 1995), the method used to freeze the embryos (Van der Elst et al., 1995), embryo quality prior to freezing (Hartshorne et al., 1990; Schalkoff et al., 1993), extent of embryo damage after thawing (Edgar et al., 2000) and resumption of post-thaw blastomere divisions (Van der Elst et al., 1997).

3.1. Developmental stage of embryos and the outcome of FET

Embryos have been successfully cryopreserved at zygote (Testart et al., 1986; Cohen et al., 1988), cleavage (Lassalle et al., 1985), and blastocyst stages (Cohen et al., 1985), using different freezing protocols either with dimethyl-sulphoxide (DMSO) (Mohr and Trounson, 1985), 1,2-propanediole (PROH) (Lassalle et al., 1985) or glycerol (Cohen et al., 1985) as cryoprotective agents.

3.1.1. Cryopreservation of zygotes

Cryopreservation of zygotes is usually accomplished using the slow freezing and quick thawing protocol with PROH and sucrose as cryoprotective agents (Testart et al., 1986). Cryopreservation at the zygote stage does not rely on a quality assessment, rather all supernumerary fertilised oocytes are frozen. The freezing of zygotes should be initiated before syngamy (20–22 hours after insemination) because freezing may cause irreversible disruption of the spindle (Balakier et al., 1993). After thawing the zygotes are considered as survived if they appear intact, with clear cytoplasm and no ZP breaches. A majority of studies have reported a high (≥70%) survival rate for zygotes (Table II). The thawed zygotes are cultured for 24 hours before transfer and the IRs ranging between 10–20% have been reported for embryos derived from frozen zygotes (Table II). The reasons for variations between the IRs of different studies are difficult to explain but may be related to the zygote freezing and thawing protocols used.

3.1.2. Cryopreservation of cleavage stage embryos

Cryopreservation of embryos on days 2 and 3 is clearly the most frequently used method in IVF. Performing cryopreservation of cleaved embryos provides one major advantage over zygote freezing, namely, only the best embryos can

be selected for storage. The cleavage stage embryo cryopreservation is routinely carried out using PROH and sucrose as cryoprotective agents and applying slow freezing and quick thawing protocol (Lassalle et al., 1985). Some authors have, however, indicated better results after cryopreservation with DMSO rather than PROH (Van der Elst et al., 1995).

Survival of cleavage stage embryos. Embryos are survived if they keep at least half of their initial number of blastomeres intact after thawing (Mandelbaum et al., 1998). The survival rates between 50 and 80% have been reported for cleavage stage embryos (Table II). A higher survival rate has been demonstrated for morphologically normal embryos with no fragments and equally sized blastomeres (Mandelbaum et al., 1987; Testart et al., 1987; Karlström et al., 1997). The survival of embryos is also highly dependent upon the number of blastomeres. A decreasing proportion of embryos with all blastomeres survived have been found with increasing cell number (Hartshorne et al., 1990). According to the authors the survival of 2–8-cell embryos is inversely related to the total surface area of all blastomeres. In agreement with the view that embryo survival decreases with increasing cell number, it has been shown that day 2 embryos survive freezing and thawing more frequently than day 3 embryos (Lassalle et al., 1985). Several studies have evaluated the impact of developmental stage of embryos on their post-thaw survival. Senn et al. showed better survival rate for zygotes (80%) than for day 2 embryos (72%) (Senn et al., 2000). Opposing results were revealed in Kattera's study, where supernumerary cleavage stage embryos frozen on day 2 had a higher rate of survival upon thawing (74%) than frozen zygotes (64%) (Kattera et al., 1999). However, in the study by Horne et al. similar survival rates were demonstrated for zygotes (74%) and day 2 embryos (77%) (Horne et al., 1997).

Implantation potential of frozen-thawed cleaved embryos. The implantation and pregnancy rates from several studies are shown in Table II. The results gathered over 10 years by a French group analysing 4 590 FETs, revealed a clinical PR of 16%, IR of 8% and delivery rate of 12% (Mandelbaum et al., 1998). In another extensive study that included data from 3 570 FETs the PR and IR were 16% and 9%, respectively (Wang et al., 2001). In that study a reduced PR was observed with increasing female age. The pregnancy and implantation rates for women aged ≤40 years (16%; 10%) were markedly better than those for women aged >40 years (8%; 4%). Previous studies comparing the results of FET between zygote and cleavage stage embryo freezing have come up with conflicting results. The cumulative PR following one fresh and two frozen ETs have been found to be similar for patients having all spare embryos cryopreserved either at zygote (40%) or cleavage stage (41%) (Horne et al., 1997). On the contrary, in another study, a higher cumulative PR after freezing of zygotes (56%) rather than cleavage stage embryos (39%) was found (Senn et al., 2000). Studies have also demonstrated that a better embryo

morphology and a faster blastomere cleavage rate are associated with improved PR after FET (Schalkoff et al., 1993; Kondo et al., 1996; Karlström et al., 1997; Edgar et al., 2000; Check et al., 2001; Tiitinen et al., 2001). Additionally, embryos can be cultured for 24 hours after thawing, and only those embryos that have undergone blastomere cleavage can be selected for transfer. Using this approach a higher PR after FET have been achieved (Van der Elst et al., 1997).

Table II. The results of embryo cryopreservation

Developmental stage	**Survival rate (%)**	**Pregnancy rate (%)**	**Implantation rate (%)**	**Reference**
Zygote	76/82 (93)	5/27 (19)	–	(Fugger et al., 1988)
Zygote	1 377/2 039 (68%)	128/449 (29)	–	(Veeck et al., 1993)
Zygote	(87)	(24)	–	(Miller and Goldberg, 1995)
Zygote	830/1 077 (77)	52/293 (18)	–	(al-Hasani et al., 1996)
Zygote	262/297 (88)	–	31/196 (16)	(Macas et al., 1998)
Zygote	657/724 (91)	80/189 (42)	(19)	(Damario et al., 1999)
Zygote	25/41 (61)	5/17 (29)	6/25 (24)	(Cohen et al., 1988)
Cleavage	44/57 (77)	6/30 (20)	6/44 (14)	
Zygote	277/494 (56)	26/112 (23)	27/252 (11)	(Demoulin et al., 1991)
Cleavage	231/492 (47)	9/110 (8)	9/191 (5)	
Zygote	96/129 (74)	11/44 (25)	–	(Horne et al., 1997)
Cleavage	79/102 (77)	4/38 (11)	–	
Zygote	(64)	(15)	–	(Kattera et al., 1999)
Cleavage	(74)	(23)	–	
Zygote	804/1 000 (80)	64/329 (19)	83/787 (11)	(Senn et al., 2000)
Cleavage	438/610 (72)	21/192 (11)	26/439 (6)	
Cleavage	10 333/14 222 (73)	754/4 590 (16)	864/10 333 (8)	(Mandelbaum et al., 1998)
Cleavage	4 363/5 572 (78)	–	463/4 720 (10)	(Edgar et al., 2000)
Cleavage	6 975/10 075 (69)	(16)	631/6 965 (9)	(Wang et al., 2001)
Blastocyst	216/289 (75)	(21)	(9)	(Menezo et al., 1992)
Blastocyst	1 033/1 239 (83)	112/516 (22)	138/1 033 (13)	(Kaufman et al., 1995)

3.1.3. Cryopreservation of blastocysts

Cryopreservation of blastocysts is yet another possibility to store super-numerary embryos for future use. The first pregnancies following transfer of frozen-thawed blastocysts were achieved in 1985, using glycerol as the cryoprotectant (Cohen et al., 1985; Fehilly et al., 1985). This method was quickly abandoned, since suboptimal culture conditions allowed only a small fraction of human zygotes to develop up to blastocyst stage. Recent progress in growing embryos in sequential culture media has increased the blastocyst

formation rate to >50% (Gardner and Lane, 1998), and revived interest in the freezing of blastocysts. Survival rates of ≥75% and PRs of ~20% have been reported for frozen-thawed blastocysts (Table II).

3.2. Chromosomal abnormalities in frozen-thawed embryos

Only a few studies have addressed the possible impact of the cryopreservation on the formation of chromosomal abnormalities in preimplantation embryos (Table I). In the study of Laverge et al. an elevated incidence of abnormalities for chromosomes 1, X and Y was demonstrated in embryos that had survived freezing and thawing but did not cleave further within the following 24 hours after thawing (Laverge et al., 1998). In that study only 12 (20%) of the 63 screened embryos were found to be uniformly diploid. Similar results were also obtained in another previous study (Iwarsson et al., 1999). By studying chromosomes 15, 16, 17, 18, X and Y a high degree of chromosomal abnormalities was revealed in frozen-thawed human embryos exhibiting good morphology. Only ~25% of the embryos had normal number of the chromosomes tested, while the majority of embryos were genetically abnormal (Iwarsson et al., 1999). In a recent study, spontaneous blastomere fusion occurring in 3% of thawed embryos was demonstrated to lead to polyploidy and chromosomal mosaicism (Balakier et al., 2000). The precise timing of freezing is critical in cryopreservation of cleavage stage embryos as embryos frozen during cellular divisions might have serious problems with correct partitioning of chromosomes into the daughter cells (Balakier et al., 1991).

III. AIMS OF THE STUDY

The objective of the study was to evaluate the importance of various embryological parameters on the success of fresh and frozen embryo transfers. The specific aims were:

- To study the effects of oocyte and spermatozoa on early embryonic development (Study I)
- To investigate whether zygote morphology (Study II) and early cleavage of embryos (Study III) could be used to predict the success rate of eSET
- To evaluate the impact of developmental stage of embryos on their post-thaw survival and the pregnancy outcome following FET (Study IV)
- To examine the effect of cryopreservation on the formation of chromosomal abnormalities in preimplantation embryos (Study V)

IV. SUBJECTS AND METHODS

1. Subjects and study design

Studies were conducted in the Infertility Clinic of the Family Federation of Finland in Helsinki from 1999 to 2002. In study I, the effects of oocytes and spermatozoa on early embryonic development were investigated. For this purpose, 59 ovum donation (OD) cycles with oocytes shared between 118 recipient couples undergoing IVF between 1992 and 2001 were analysed. The oocyte donors were unpaid volunteers <37 years of age. The mean age ± standard deviation (SD) of oocyte donors was 29.4 ± 4.1 years (range 21–36 years) and recipients 33.8 ± 4.9 years (range 23–49 years). Oocyte donation provided a unique model in which oocytes from a single donor were randomly divided between two recipient couples and were inseminated by sperm from two men.

The data in studies II and III were collected in collaboration with Helsinki University Central Hospital between 1999 and 2002. In these studies the analysis of factors influencing the PR in eSET procedures was performed. The possibility whether the zygote morphology could be used to predict the PR following eSET was evaluated in study II. The study involved 191 patients undergoing either IVF (n = 134) or ICSI (n = 57), with an average age of 33.8 ± 4.0 years (range 24–43 years). In study III, the relationship between early cleavage of embryos and the success of eSET was retrospectively examined. From the analysed 178 eSET procedures, 133 and 45 were IVF and ICSI procedures, respectively. The average age of women was 33.4 ± 4.0 years (range 23–42 years).

Studies IV and V dealed with the factors influencing the outcome of FET. In study IV, the impact of developmental stage of embryos on their post-thaw survival and the PR following FET were elucidated. The analysis included all patients (n = 875) undergoing IVF (n = 697) or ICSI (n = 322) treatment from 1993 to 2001 with a FET (n = 1657) between 1997 and 2001. The average age for all patients was 34.0 ± 4.3 years (range 22–44 years). The objective of study V was to examine the effect of cryopreservation on the formation of chromosomal abnormalities in preimplantation embryos. To this end, the chromosomal constitutions of cryopreserved embryos were assessed using FISH technique. Twenty-eight patients undergoing IVF or ICSI procedure between 1997 and 1999 provided 61 frozen zygotes and cleaved embryos for the study after informed consent was obtained from each couple. Twenty-one couples underwent IVF and provided 48 embryos while 7 patients underwent ICSI and provided 13 embryos. The average age of patients donating the embryos was 34.4 ± 3.5 years (range 29–43 years).

2. Methods

2.1. Ovarian stimulation protocol (Studies I–III)

The pituitary down-regulation was performed using the long protocol with gonadotropin-releasing hormone agonist (Synarela; Syntex Nordica AB, Södertälje, Sweden). The suppression was followed by ovarian stimulation with human menopausal gonadotropins (Humegon; Organon, Oss, the Netherlands, or Pergonal; Laboratories Serono S.A., Aubonne, Switzerland), highly purified FSH (Follegon; Organon, or Fertinorm HP; Laboratories Serono S.A.) or recombinant FSH (Puregon; Organon, or Gonal-F; Laboratories Serono S.A.). Human chorionic gonadotropin (Pregnyl; Organon, or Profasi; Laboratories Serono S.A.) was administered when two or more follicles reached the size of ≥17 mm in diameter and OPU was performed 36 hours later.

2.2. Semen analysis and preparation (Studies I–III)

In semen analysis, standard sperm characteristics as concentration, motility and morphology were evaluated. Sperm concentration was determined with the use of a Makler® counting chamber (Sefi Medical Instruments, Haifa, Israel). Motility was expressed as the percentage of progressively motile spermatozoa according to WHO guidelines (WHO, 1999). Sperm morphology was estimated according to Tygerberg strict criteria (Kruger et al., 1988) on air-dried smears, fixed and stained by a modified Papanicolaou stain (Spermac®; Fertipro, Beernum, Belgium). In study I, all patients were divided into three groups according to the proportion of morphologically normal sperms (Kruger et al., 1988). Patients in the first group possessed <4% of morphologically normal sperm cells, patients in the second group had 4–14% of normal sperm cells, and patients in the third group had >14% of normal sperm cells. The semen sample was prepared by a 45–90% discontinuous gradient centrifugation method using Percoll® (Pharmacia, Uppsala, Sweden) or PureSperm (Nidacon International AB, Gothenburg, Sweden). The pellet was collected from the bottom of the 90% layer, washed once with Universal-IVF medium (U-IVF, Medi-cult, Copenhagen, Denmark) and resuspended.

2.3. IVF and ICSI (Studies I–III)

The quality of COCs were evaluated as having expanded or compact cumulus and corona (Study I). Oocytes with well-expanded cumulus were equally divided between two recipient couples of the same oocyte donor. In normal IVF, oocytes were inseminated 5–6 hours after OPU with ~25 000 progressively

motile spermatozoa per oocyte in 1 ml of Universal-IVF medium in Falcon single well dishes (Becton Dickinson, San Jose, CA, USA). ICSI procedure was performed as previously described (Van Steirteghem et al., 1993). Briefly, the cumulus cells were removed from COCs by pipetting them in hyaluronidase solution (80 IU/ml) (H-4272; Sigma). The maturational stage of oocytes was evaluated and only metaphase II oocytes were injected 5–6 hours after OPU. Oocytes were placed in droplets of HEPES (Gibco) buffered Universal-IVF medium and a single spermatozoon was injected directly into the ooplasm. Normally fertilised oocytes manifested two PNs and PBs 16–18 hours after insemination or ICSI. Zygotes were cultured in Universal-IVF medium for 24 or 48 hours before being transferred or cryopreserved. In studies II–V, IVF and ICSI procedures were combined, while in study I, only IVF procedures were analysed.

2.4. Evaluation of zygote morphology (Study II)

In evaluation of zygote morphology the localisation and the number of NPB and the existence of cytoplasmic halo were studied 16–18 hours after insemination or ICSI (Figure 1). The two classification systems used for pronuclear morphology have been detailed in original study II and depicted in Figure 2. After examination, zygotes were cultured in separate drops of culture medium.

2.5. Assessment of embryo quality (Studies I–III)

Embryos possessing 2 cells at 25–27 hours post-insemination were designated as EC embryos and those that had not yet cleaved were classified as NEC embryos (Study III). Cleavage stage embryo quality was evaluated 42–46 hours after insemination considering the number of blastomeres, the degree of fragmentation, the uniformity of blastomeres and the presence of MNB. Embryo morphology was scored as follows: grade 1, no fragments and equal blastomeres; grade 2, <20% fragmentation; grade 3A, unequal blastomeres and/or 20–35% fragmentation; grade 3B, unequal blastomeres and/or 35–50% fragmentation and grade 4, >50% fragmentation. In study III, the degree of fragmentation was expressed as a percentage of the perivitelline space occupied by cytoplasmic fragments. Embryos were considered evenly cleaved when the difference in size between blastomeres was ≤10%. Comparison of embryo parameters (morphology and number of blastomeres) between two recipient couples of the same oocyte donor allowed to distinguish the influences of oocytes and spermatozoa on early embryonic development (Study I). The percentages of good morphology embryos (grades 1 and 2), embryos with ≥3 (Study II) or ≥4 blastomeres (Study III) 42–46 hours after insemination or ICSI

and embryos with MNB were calculated for each class of zygotes (Study II) and for EC and NEC embryos (Study III).

2.6. Fresh ET (Studies I–III)

A maximum of two embryos were selected for transfer in study I, while studies II and III comprised exclusively of eSETs. The patients were deemed eligible for eSET if they were ≤37 years old, and were in their first or second IVF or ICSI treatment. Other indications for eSET were: patient's wish to avoid multiple pregnancies, previous successful IVF or ICSI treatment and risk of ovarian hyperstimulation syndrome. eSET was feasible when good quality embryo with mononucleated blastomeres and ≤20% of fragmentation was available for transfer. Selection of the embryo for transfer was based on embryo quality on day 2 or 3 and the whole process was not influenced by zygote morphology and early cleavage. Vaginal progesterone was used for luteal support. A positive serum hCG test (>10 mIU/mL) conducted 16 days after embryo transfer confirmed pregnancy and the clinical pregnancy was documented by the presence of a gestational sac on transvaginal sonography approximately three weeks later. The transfer of single embryo in studies II and III provided an excellent opportunity to examine the effects of various embryological parameters on the success of IVF.

2.7. FET (Study IV)

Identical slow freezing and quick thawing protocol with PROH (Sigma, USA) and sucrose as cryoprotectants was used for cryopreservation of both zygotes and cleaved embryos (Lassalle et al., 1985). The developmental stage of embryos at freezing was dependent on the day of OPU as no freezing is performed on Saturdays and Sundays in our clinic. All supernumerary zygotes were cryopreserved, while only good quality spare embryos of grades 1-3A were selected for freezing. Cleaved embryos were classified after thawing as follows: fully intact, partially damaged (≥50% of cells survived) and degenerated (<50% of cells survived). Zygotes were cultured for 24 hours before transfer, while the cleaved embryos were mostly transferred on the day of thawing. A maximum of two embryos were transferred and the PRs were compared between three different cryopreservation strategies utilising either zygote, day 2 or day 3 embryo freezing.

2.8. FISH on embryos (Study V)

Two groups of embryos were analysed using FISH method for chromosomes 13, 16, 18, 21, X and Y, as described extensively in the original communication V. Study group embryos frozen at zygote or 2-cell stage (n = 29) were cultured *in vitro* post-thaw until they reached 4–6-cell stage, after which their chromosomal constitutions were assessed. Control group embryos frozen at 4–6-cell stage (n = 32) were analysed immediately after thawing in order to exclude any post-thaw effect. Based on the chromosomal constitutions of blastomeres, embryos were allocated to four classes: normal embryos; aneuploid embryos; mosaic embryos and chaotic embryos. Aneuploid embryos possess either monosomy or trisomy in all blastomeres indicating that the genetic aberration was already present in gametes. Mosaic embryos possess chromosomally abnormal cells along with normal ones, while chaotic embryos have only abnormal cells. However, blastomeres of chaotic embryos have different chromosomal abnormalities. Contrary to the aneuploidy, the chromosomal aberrations of mosaic and chaotic embryos emerge during embryo development. Therefore, the effect of cryopreservation on the formation of chromosomal defects in early embryos can be evaluated by comparing the proportions of chromosomally abnormal embryos in study and control groups.

2.9. Statistical analysis (Studies I–V)

Means were given with SD and were compared using a two-tailed unpaired Student's t-test. Correlations were estimated using linear regression analysis and were characterised by Pearson's correlation coefficients (r). Cross-tabulated data were compared with the χ^2 test. The investigation of factors influencing the embryo quality (Study I) was carried out using analysis of variance (ANOVA) as implemented in the mixed procedure of the SAS system, release 8.1. (SAS, 2001). The independence of various factors known to affect the PR after eSET (Study III) was tested applying generalised linear analysis using SAS system (Release 8.1.) procedure GENMOD, binominal distribution and standard logit link. The significance level was considered at $P < 0.05$.

V. RESULTS AND DISCUSSION

1. Results

1.1. The effects of gametes on early embryo development (Study I)

The study on the factors involved in embryo development included 646 embryos. Pearson's correlation analysis revealed a strong correlation ($r = 0.67$, $P < 0.0001$) between the embryo morphology of the first recipient couple and that of the second recipient couple of the same OD procedure. A weaker correlation ($r = 0.38$, $P < 0.0005$) was found between the average blastomere number of the first recipient couple and that of the second recipient couple. In addition, mixed ANOVA demonstrated that the oocyte has a considerable effect ($P < 0.0001$) on embryo morphology and a weaker effect ($P = 0.01$) on blastomere cleavage rate. The test revealed also a significant ($P = 0.015$) effect of sperm cell on the blastomere cleavage rate but not on embryo morphology. More specifically, the sperm morphology was shown to be positively associated ($P = 0.03$) with blastomere cleavage rate. However, other sperm characteristics such as sperm concentration and progressive motility before and after sperm preparation did not influence the blastomere cleavage rate. Furthermore, there was no correlation between sperm characteristics and embryo morphology.

1.2. Zygote morphology and the success rate of eSET (Study II)

The presence of halo was checked in 764 zygotes from 105 eSETs and a clear cortical cytoplasm was observed in 67.7% of zygotes. In the cohort of halo-positive zygotes, some of the zygotes had symmetrical halo located homogeneously along the cortex of cytoplasm whereas others exhibited asymmetrical halo located predominantly on one side of the PNs. In the group of halo-positive zygotes there were more ($P < 0.05$) good quality embryos (60.9%) than in the group of halo-negative zygotes (52.2%). To the contrary, the proportion of embryos having ≥3 blastomeres was similar among halo-positive (53.2%) and -negative (51%) zygotes. The implantation and delivery/ongoing pregnancy rates were also found to be comparable for halo-positive (29.9%, 23/77 and 24.7%, 19/77) and -negative (25%, 7/28 and 21.4%, 6/28) zygotes.

A total of 764 zygotes from 105 eSETs were evaluated using the scoring system of Scott and Smith (Figure 2) (Scott and Smith, 1998). Of all zygotes, 44.1% (n = 337), 36.4% (n = 278) and 19.5% (n = 149) belonged, respectively, to classes I, II and III. The elevated ($P < 0.005$) proportions of embryos having ≥3 blastomeres were found in classes I (57.3%) and II (54%) than in class III (38.9%). However, the fractions of good morphology embryos were similar in all classes. Although the slightly increased implantation and delivery/ongoing

pregnancy rates after eSET were shown in classes I (29.2%, 14/48 and 22.9%, 11/48) and II (31.7%, 13/41 and 29.3%, 12/41) compared to class III (18.8%, 3/16 and 12.5%, 2/16), the difference was not significant.

All zygotes (n = 1520) from 191 eSETs were scored according to the classification system of Tesarik and Greco (Figure 2) (Tesarik and Greco, 1999). For 1178 (77.5%) zygotes single NPB pattern was attributed whereas for 342 (22.5%) zygotes two or more patterns were simultaneously described. For calculations only those embryos classified to a single NPB pattern were used. There were no differences in the proportions of embryos having ≥3 blastomeres, good morphology or MNB between pattern 0 and non-pattern 0 groups (Table III). Similar to embryo quality, the two groups demonstrated no differences in implantation and delivery/ongoing pregnancy rates.

Table III. Zygote scoring according to Tesarik's classification system

PN pattern	**n**	**Embryos with ≥3 blastomeres (%)**	**Embryos with good morphology (%)**	**Embryos with MNB (%)**	**Implantation rate (%)**	**Delivery/ ongoing PR (%)**
0	440	65	63.9	18.6	33.9 (19/56)	28.6 (16/56)
1	87	62.1	83.3[c]			
2	135	53.3[a]	62.1			
3	112	69.6	68.8	18.8	31.8 (28/88)	26.1 (23/88)
4	36	52.8[b]	65.6			
5	368	69	71.5[d]			

[a]P < 0.05 when compared to the 0, 3 and 5 patterns; [b]P < 0.05 when compared to the 5 pattern; [c]P < 0.05 when compared to the 0, 2, 3, 4 and 5 patterns and [d]P < 0.05 when compared to the 0 and 2 patterns

1.3. Early cleavage and the PR after eSET (Study III)

The analysis comprised of 1379 embryos from 178 eSETs. Early cleavage was observed in 26.3% of embryos. The proportion of good morphology embryos was higher (P = 0.044) among EC (50.5%) than NEC (44.2%) embryos. EC embryos possessed also more blastomeres on day 2 as the proportion of EC embryos with ≥4 blastomeres (94.1%) exceeded (P < 0.0001) that of NEC embryos (51%). In addition, the incidence of MNB was lower (P = 0.005) in EC (18.9%) than in NEC (29.6%) embryos. The transfer of EC embryos resulted in higher (P = 0.001) clinical PR (50%) when compared to NEC embryo transfers (26.4%). The mean degree of fragmentation was found to be similar in EC and NEC transferred embryos, but EC embryos possessed more blastomeres (4.0 vs. 3.7; P = 0.003), and also exhibited evenly sized blastomeres more frequently

(55.6% vs. 36.2%; P = 0.011) than NEC embryos. After 4-cell stage ETs, a better (P = 0.025) clinical PR was achieved with EC (50.7%) than with NEC embryos (32.5%). A higher (P < 0.0001) clinical PR was observed after eSET with evenly cleaved EC embryos (55%) than unevenly cleaved NEC embryos (20.9%). Also, a better (P = 0.018) clinical PR was seen following transfer of unevenly cleaved EC (43.8%) than NEC embryos.

The analysis of all embryological factors determining the success of eSET demonstrated a clear positive correlation (P = 0.001) between the early cleavage of transferred embryos and the establishment of the pregnancy, as more EC embryos were transferred to the pregnant (56.3%) than to non-pregnant women (31.6%). The proportion of evenly cleaved transferred embryos was also higher (P = 0.014) among pregnant (56.3%) than non-pregnant (37.2%) women. Further analysis indicated that the early cleavage as well as the regularity of blastomeres determine independently the developmental potential of embryos.

1.4. Developmental stage of embryos and the results of FET (Study IV)

From the thawed embryos (n = 4006), 562 were zygotes and 3444 cleaved embryos. A majority (91%) of the cleavage stage embryos were frozen on day 2 (n = 3133) and the remaining 311 on day 3. The highest (P < 0.0001) survival rate was observed for zygotes (86.5%), followed by day 2 (61.7%), and day 3 (43.1%) embryos. The proportion of partially damaged embryos was lower (P = 0.002) on day 2 (17.6%) than on day 3 (24.8%). A lower (P < 0.0001) percentage of degenerated embryos was found for zygotes (13.5%) than for day 2 (20.7%) and day 3 (32.1%) embryos. An inverse correlation (r = –0.9; P = 0.003) was found between the proportion of intact zygotes or cleaved embryos and the total number of cells/blastomeres. The fraction of partially damaged embryos showed a positive correlation (r = 0.8; P = 0.014) with the cell number, while the incidence of degenerated embryos did not show a relationship with the number of cells.

Embryos were transferred in 95.7% of all thaw cycles (n = 1657). With the transfer of 1.7 embryos on average, the overall clinical pregnancy and implantation rates were 20.7% (329/1586) and 14.2% (373/2635), respectively. All FETs resulted in 262 deliveries (16.5% per embryo transfer) and the birth of 291 children (11% per embryo transferred). There were no differences between three cryopreservation strategies utilising zygote, day 2 or day 3 embryo freezing in the mean age and the average number of embryos transferred, nor in the clinical pregnancy or implantation rates. An elevated miscarriage rate was observed in day 3 transfer group (45%) compared to zygotes (21.3%; P = 0.049) and day 2 (18.3%; P = 0.004) embryos. The delivery rate for frozen zygotes (15.8%) was similar to that observed for day 2 embryos (17.2%). Although lower delivery rate was found for day 3 transfers (10.1%) than for zygotes and

day 2 embryos, these differences were not significant. The birth rate was also comparable in all three groups. The similar efficacy (the birth rate per embryo thawed) was observed for zygote (7.1%) and day 2 (7.6%) embryo freezing. The efficacy for day 3 (4.2%) embryo freezing was, however, lower ($P = 0.027$) when compared to day 2 embryos.

1.5. Chromosomal defects in cryopreserved embryos (Study V)

FISH was performed on study (n = 29) and control (n = 32) group embryos using probes for chromosomes (13, 16, 18, 21, X and Y) (Figure 3). The analysis was successfully accomplished on 85% of 305 blastomeres. The women's age, the proportions of IVF and ICSI embryos and the prevalences of good quality embryos were similar in both groups. Although there were fewer normal embryos in study (20.7%) than in control (31.3%) group the difference was not significant. The prevalences of aneuploid (10.3% vs. 12.5%) and mosaic (44.8% vs. 50%) embryos were also comparable in two groups. A higher ($P < 0.05$) proportion of chaotic embryos was observed in study (24.1%) than in control group (6.3%). Seven (11.4%) (three in study and four in control group) embryos were aneuploid: one embryo had trisomy 13, two embryos had trisomy 16, one embryo had monosomy 16, two embryos had trisomy 21 and one embryo had sex chromosome aneuploidy (XYY).

2. Discussion

2.1. The effects of gametes on early embryo development

Embryo quality is one of the most essential factors determining the success of IVF (Walters et al., 1985; Rogers et al., 1986). Several attempts have been made to elucidate the effects of gametes on embryo quality in a routine IVF program, though this approach seems to be inadequate as probably both the oocyte and sperm cell influence the embryo development (Ron-El et al., 1991; Parinaud et al., 1993; Xia, 1997; Loutradis et al., 1999; Ebner et al., 2000). In our OD program, oocytes from single donor are randomly divided between two recipient couples and inseminated by two different sperm samples, thus providing the possibility to assess the individual contributions of both gametes to embryo quality. Comparison of the variances in embryo morphology and blastomere cleavage rate between different OD cycles as well as between two recipient couples of the same OD cycle revealed that oocyte has a profound effect on embryo morphology and less of an effect on the blastomere cleavage rate (Study I). An association between oocyte and embryo quality has also been demonstrated in previous studies (Xia, 1997; Loutradis et al., 1999; Ebner et al.,

2000) and can be explained by the fact that embryo development during the first three days depends largely on the organelles, proteins and RNAs stored in oocytes (Gougeon, 1996).

In addition, an effect of sperm cell on blastomere cleavage rate was found (Study I). More detailed analysis indicated a positive relationship between the sperm morphology and blastomere cleavage rate. In other words, faster blastomere cleavage rate may be expected in patients with higher proportion of normal sperm cells than in patients with impaired sperm quality. This finding is particularly interesting in view of the results from some studies showing that blastomere cleavage rate is the most important determinant of the developmental potential of the embryo (Giorgetti et al., 1995; Ziebe et al., 1997). Several studies have so far been conducted to uncover the possible effect of sperm cell on embryo quality. Tesarik et al. showed that the sperm effect occurs at zygote stage (Tesarik et al., 2002). Other studies have demonstrated an impaired early embryo development (Ron-El et al., 1991; Parinaud et al., 1993) and a reduced blastocyst formation rate when sperm with lower quality were used in IVF (Janny and Meneso, 1994; Shoukir et al., 1998; Dumoulin et al., 2000). The cellular processes responsible for sperm effect are still poorly understood. The sperm cell is known to contribute the centrosome to the oocyte during fertilisation. The centrosome is involved in cell division and its structural and functional defects might cause disorders of fertilisation and early embryo development (Asch et al., 1995). The importance of sperm DNA packaging on embryo development cannot also be underestimated as deficiences in chromatin packaging might as well lead to incorrect PN formation and delayed cell division events (Larson et al., 2000). Both our study and other similar studies, providing evidence of sperm effect on embryo quality contradict the others indicating a lower fertilisation rate but normal embryo quality in IVF with poor quality semen (Tournaye et al., 1992; Terriou et al., 1997).

2.2. The influences of various embryological features on the success rate of eSET

2.2.1. Zygote morphology and the success rate of eSET

Two classification systems have been established for the assessment of zygote morphology (Figure 2) (Scott and Smith, 1998; Tesarik and Greco, 1999), which predictive value on cleavage stage embryo quality and pregnancy outcome following eSET was retrospectively evaluated in study II. According to the first system, the polarised NPB pattern and the existence of cytoplasmic halo are considered as good signs for embryo development (Scott and Smith, 1998). Our data revealed that embryos having both (class I) or at least one PN (class II) with polarised NPB cleaved faster than those having scattered NPB (class III). In addition, the proportion of the good morphology embryos was

higher among halo-positive than -negative embryos. These findings are in good agreement with previous studies showing a better embryo quality for halo-positive zygotes with polarised distribution of NPB (Scott et al., 2000; Zollner et al., 2002). Although IR and delivery/ongoing PR of the class III embryos (18.8%, 12.5%) were lower than those of the classes I (29.2%, 22.9%) and II (31.7%, 29.3%), these differences were not significant. Neither there were any differences in IR and PR between halo-positive and -negative zygotes. The rationale of the second classification system is that for proper embryonic development interpronuclear synchrony is more important than the actual polarisation of NPB within individual PN (Figure 2) (Tesarik and Greco, 1999). Using Tesarik's classification system, we were unable to find any association between zygote morphology and either cleavage stage embryo quality or IR.

There are several probable reasons for discrepancy between the pregnancy results of our study and those of others (Scott and Smith, 1998; Montag and van Der Ven, 2001). At first, in those studies more than two zygotes with appropriate morphology were selected for ET. In our study, only single embryo was chosen for transfer based solely on embryo quality. eSET was performed if good morphology embryo was available for ET. If this condition was not met, two embryos were transferred. This limitation means that in eSETs good quality class III, halo-negative and non-pattern 0 embryos were transferred and it seems that the viability of these embryos is not compromised when compared to others. In addition, the scoring system of Scott and Smith includes the evaluation of early cleavage, though its association with positive outcome of IVF has been suggested (Shoukir et al., 1997; Sakkas et al., 1998). Collectively, the results of the study II support the concept that NPB polarisation (at least in one PN) may be related to successful embryo development. Therefore it could reasonably be assumed that the evaluation of zygote morphology might be beneficial in countries with strict embryo protection laws. In these countries, only as many zygotes are cultured as are planned to be transferred and therefore the selection of good prognosis zygotes for culture can supposedly improve the quality of transferred embryos. In other countries, the assessment of zygote morphology would not provide any additional advantage in embryo selection.

2.2.2. Early cleavage and the PR after eSET

Recently, evidence has been presented indicating the potential value of early cleavage in embryo selection (Shoukir et al., 1997; Sakkas et al., 1998). The assessment of early cleavage have quickly found its way to the clinical use, though the scientific basis for the change is not fully established. Furthermore, the possibility of using early cleavage in embryo selection for eSET has remained totally unexplored. The analysis of pregnancy results of eSETs in study III demonstrated a better IR after transfer of EC (50%) than NEC (26.4%) embryos. To exclude the possibility that the higher IR achieved with EC

embryos could be caused by the fact that these embryos contained more blastomeres at the time of ET than NEC embryos, we subsequently analysed only the 4-cell stage embryo transfers. Again, a better IR was achieved with EC (50.7%) than with NEC (32.5%) embryos. The high IR for EC embryos reported in study III that has previously demonstrated only for blastocysts (Gardner et al., 1998) would imply that embryo selection could be successfully accomplished at cleavage stage making the extended culture and blastocyst transfer redundant. The further analysis of possible factors predicting the success of eSET revealed that both early cleavage and regularity of blastomere divisions influence the embryo viability (Study III). These findings are in complete agreement with other studies showing the relationship between even cleavage of embryos and better capacity for implantation (Giorgetti et al., 1995; Hardarson et al., 2001). The evaluation of all embryos demonstrated that EC embryos possessed a better morphology and a lower incidence of MNB than NEC embryos, supporting the conclusions of an earlier study (Lundin et al., 2001). Taken together, the study III presents a set of findings suggesting that the evaluation of early cleavage may provide the supplementary criterion to be used in embryo selection for eSET.

2.3. Factors related to the outcome of FET

2.3.1. Developmental stage of embryos and the results of FET

Developmental stage of embryos at freezing is known to affect the results of FET, but the issue has not been addressed in a thorough manner. To the best of our knowledge, study IV is the first to compare the outcome of zygote, day 2 and day 3 embryo cryopreservations in which identical freezing and thawing protocols were used for both zygotes and cleaved embryos. Our results suggest that the developmental stage of embryos has a major impact on their survival after freezing and thawing as the best survival rate was found for zygotes (86.5%), followed by day 2 (61.7%) and day 3 (43.1%) embryos, while the opposite was found for degenerated embryos. These findings are consistent with other studies that have reported a better survival rate for zygotes than for day 2 embryos (Senn et al., 2000), and for day 2 than for day 3 embryos (Mandelbaum et al., 1987). The importance of developmental stage on embryo survival was also reinforced by our observation of a correlation between the extent of cryodamage and the number of blastomeres. It has been thought that this might be due to the increased total surface area of all cells (Hartshorne et al., 1990).

The comparison of IR and PR revealed no differences between zygote, day 2 and day 3 FETs (Study IV). In contrast, other authors have found better IR and PR after zygote than day 2 FET (Demoulin et al., 1991; Senn et al., 2000) or vice versa (Kattera et al., 1999). So far, only in one small study comprising 185 FETs a comparison has been made between the outcome of day 2 and 3

FET, and a better PR for day 2 than for day 3 FET was demonstrated (Mandelbaum et al., 1987). The differences between studies cannot be easily explained, but would be attributed to a range of factors, including the type of ovarian stimulation regimen used, the criteria applied for embryo selection for freezing, and the embryo freezing and thawing protocols applied. It is of importance that higher miscarriage rate (45%) was found in day 3 FET group than in other groups (Study IV). The rate of miscarriage has been ascertained to be higher in frozen (26.3%) than in fresh (22%) ET pregnancies (STAKES, 2002). On the other hand, the miscarriage rate for fresh day 2 and 3 ETs has been demonstrated to be similar (Dawson et al., 1995; Carrillo et al., 1998). Therefore, the elevated miscarriage rate for day 3 frozen embryos is likely to be caused by a damage during the freezing and thawing procedures. One possible explanation could be that the proportion of partially damaged embryos was the highest (24.8%) in day 3 FET group. The elevated miscarriage rate resulted in lower delivery and birth rates for day 3 FETs, but the differences from other groups were not statistically important. The overall efficacy of FET can be expressed as the birth rate per embryo thawed (Van der Elst et al., 1995). The efficacy of cryopreservation was lower in day 3 (4.2%) than day 2 (7.6%) group. A better birth rate per embryo thawed was also reported for zygotes (7.1%) than for day 3 embryos, though this trend did not reach significance. The low survival rate and elevated miscarriage rate were both responsible for the reduced overall efficacy for day 3 FET when compared to zygotes and day 2 embryos. In our study PROH was used in cryopreservation of zygotes as well as cleavage stage embryos. Some reports, however, indicate better survival and implantation rates after cleavage stage embryo cryopreservation with DMSO rather than PROH (Van den Abbeel et al., 1988; Van der Elst et al., 1995). Thus it can be speculated that the results might be different if DMSO instead of PROH was used for freezing of cleavage stage embryos.

2.3.2. Chromosomal defects in cryopreserved embryos

There is some evidence indicating a causal link between the embryo cryopreservation and the generation of chromosomal abnormalities (Laverge et al., 1998; Iwarsson et al., 1999; Balakier et al., 2000). Considering the scarce information available, the objective of the study V was to investigate the effect of cryopreservation on the formation of chromosomal defects in early embryos. This was accomplished by performing FISH on two groups of embryos. The embryos in study group were frozen either at zygote or 2-cell stage and were cultured after thawing up to 4–6-cell stage before analysis. Although the embryos in control group were also frozen, they were analysed immediately after thawing. This ensures that the chromosomal constitutions observed in these embryos were unaltered compared to the situation prior to freezing (Munne et al., 1997). A comparison of the proportion of chromosomally abnormal embryos between

study and control groups would thus demonstrate the role of cryopreservation in the generation of the chromosomal defects in embryos. A fewer number of normal embryos was observed in study (20.7%) than in control (31.3%) group, but this decrease was not statistically significant (Study V). In addition, the proportion of chaotic embryos was higher in study (24.1%) than in control (6.3%) group. These findings are in accordance with previous studies showing elevated prevalence of chromosomal defects in frozen-thawed embryos (Laverge et al., 1998; Iwarsson et al., 1999; Balakier et al., 2000). An increased level of chromosomal defects may be an additional factor that would lower the developmental competence of frozen-thawed embryos when compared to fresh embryos (Aytoz et al., 1999). It has been argued that zygote freezing at syngamy and embryo freezing during cellular cleavage may pose a risk to the integrity of the spindle (Pickering et al., 1990), thus supposedly leading to chromosomal abnormalities (Balakier et al., 1991; Balakier et al., 1993). In study V, the zygotes were frozen before syngamy (20–22 hours post-insemination), though it cannot be excluded that some zygotes had already passed beyond the optimal time for freezing. It is also common practice to freeze all cleavage stage embryos together on the second or third day after insemination or ICSI. This heterogeneous group is likely to contain embryos in various stages of blastomere division implying that some of the cleavage stage embryos may be more prone to freezing damage than others.

VI. CONCLUSIONS

Using the data from our donor oocyte-sharing program from the years 1992–2001, the individual contributions of gametes to early embryo development were investigated (Study I). A marked effect of the oocyte on both embryo morphology (fragmentation and blastomere uniformity) and blastomere cleavage rate was demonstrated. In addition, an effect of sperm cell on blastomere cleavage rate was found. More specifically, sperm morphology rather than sperm count or progressive motility was positively associated with the blastomere cleavage rate. According to our results, the measured sperm characteristics were unrelated to embryo morphology.

The influences of various embryological features on the success of implantation were studied in IVF and ICSI procedures performed in our clinic from 1999 to 2002. The strength of our work is that only a single embryo was transferred to all patients participating in these studies providing the excellent possibility to evaluate the associations between embryo quality and the chance of pregnancy. The zygote morphology was found to be predictive of normal embryo development, but did not influence the implantation rate after eSET (Study II). While early cleavage of zygotes proved to be an important determinant of both normal embryo development and embryo viability (Study III). The comparison of IRs between EC and NEC single embryo transfers revealed the potential value of early cleavage in embryo selection for eSET.

The effects of the developmental stage of embryos on their post-thaw survival and the pregnancy outcome after frozen embryo transfer were scrutinised on a cohort of patients who attended our clinic for FET between 1997 and 2001 (Study IV). Our results support the view that the developmental stage of embryos has a profound effect on their post-thaw survival, as the best survival was observed for zygotes, followed by day 2 and 3 embryos. To the contrary, the developmental stage seems to be unrelated to the pregnancy, implantation, delivery and birth rates after FET. The elevated miscarriage rate observed in day 3 group compared to zygotes and day 2 embryos was likely caused by the damage during freezing and thawing procedures. The low survival rate and elevated miscarriage rate were both responsible for a reduced overall efficacy for day 3 FET when compared to zygotes and day 2 embryos.

In study on the chromosomal defects in frozen-thawed embryos, further support was given to the hypothesis that cryopreservation may induce chromosomal abnormalities in frozen-thawed embryos (Study V). A higher incidence of chromosomal defects was found in embryos that had undergone cellular divisions after thawing than embryos analysed immediately after thawing. The induced chromosomal defects may impair the viability of cryopreserved embryos.

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