On embryo implantation

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The studies described in this thesis were performed at the Division of Reproductive Medicine, Department of Obstetrics and Gynaecology, VU medical center, Amsterdam, the Netherlands.

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On embryo implantation

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prof.dr. C.B. Lambalk

‘De natuur kent het grote geheim en glimlacht.’

Victor Hugo

Content

1 General introduction

General introduction

Although having children is often regarded as something natural, at present a rising number of couples are calling on assisted reproductive techniques to fulfil their desire to have children. In the Netherlands, one out of ten couples is confronted with subfertility and the majority of these couples present themselves to a hospital for investigation of fertility, often resulting in fertility treatment; ranging from ovulation induction to intra-cytoplasmatic-sperm-injection (ICSI)[1].
One of the reasons the incidence of fertility treatment in the Netherlands is so high, is because women tend to have their children later in life, partially as a result of education and career planning. The average age of having a first child has over the years risen to 29.4 years[2], making Dutch women the oldest mothers in the world. By postponing pregnancy, the risk of infertility increases and therefore the need for assisted reproductive techniques[3]. In 2007, around 9100 in-vitro-fertilisation (IVF) and 7000 ICSI treatments were performed in the Netherlands.
IVF has gone through rapid developments over the past forty years. When in 1978 Louise Brown, the first 'test-tube baby'[4], was born, the IVF-procedure was very different from today's IVF-treatment. Patients with blocked tubes were monitored throughout their natural cycles by frequent measurements of estradiol in 24-hour urine samples. When estradiol levels indicated that the follicle was mature and ovulation was to be expected, the patient was admitted for retrieval of the one, or maybe two, oocyte(s). The oocyte retrieval was performed by laparoscopy under general anesthesia[5;6].
After percutaneous sonography was introduced in IVF-treatment, ultrasonography had a major role in the monitoring of the treatment cycle. For a short period oocyte retrieval was also performed under percutaneous transvescular ultrasonographic guidance. After the development of the transvaginal ultrasound transducer cycle monitoring and oocyte retrieval could be done transvaginally and in an out-patient setting[7].
At first, IVF was performed in natural cycles; in the 1980s it became more common to induce multiple follicle growth using gonadotrophins or clomiphene citrate[6]. All facilities and staff had to be available at all times since the occurrence of the LH-surge could not be predicted accurately, unless human chorionic gonadotrophin was administered to induce final maturation of the oocyte and ovulation.
After introduction of gonadotrophin release hormone (GnRH)- agonists, it became possible to postpone, or even schedule, oocyte retrieval and to stimulate the ovaries for multiple follicle growth without the risk of a premature LH-surge. Therefore, creating the possibility to retrieve a higher number of oocytes and increasing the chance of fertilisation, the development of (good) embryos and of

a subsequent pregnancy.
Gradually, the indication for IVF became broader: it was demonstrated to be beneficial in couples with oligospermia[8] and unexplained infertility. The coincidental discovery of intra cytoplasmatic sperm injection (ICSI)[9] created a whole new era of assisted reproduction. Because only a few sperm cells were needed for fertilisation, it became possible to treat couples with severe oligospermia by treating the woman and performing ICSI on the retrieved oocytes.
The embryo transfer has also gone through several changes over the same period. With the idea to have optimal positioning of the uterus in anteversion flexion embryo transfer was performed with the patient positioned in knee-chest position[10]. Unsure whether gravity might get hold of the embryos before they could take hold in the uterus the patient was admitted to the hospital for 24 hours to remain in horizontal position, to prevent the transfered content from 'falling out'. Gradually, it was realised that transfers could just as well be performed in lithotomy position and the patient's position at transfer was changed. By now the period of hospitalisation after transfer has become shorter and is, or soon will be, finally abandoned completely. Different types of transfer catheters have been used for embryo transfer[11] and ultrasonography has also found its use in the embryo transfer by making it possible to visualise the catheter and the content of the catheter[12], creating a possibility of more precise and controlled handling of the catheter at the transfer.
The IVF patients of the seventies and eighties underwent frequent blood and urine sampling for cycle monitoring, they were admitted for laparoscopic retrieval of hopefully one (or maybe two) oocytes and, in case of fertilisation, they were admitted once more for embryo transfer. Today's patients inject themselves with GnRH-agonist or GnRH-antagonists and FSH daily, they have cycle monitoring by ultrasound and occasionally by measurement of serum estradiol levels, they spend less than half a day in the hospital for the retrieval of a number of oocytes and half an hour for transfer of a carefully selected embryo that is performed under ultrasonographic guidance.
With all these improvements, success rates have risen to plateau levels around 25-30%. Because of high multiple pregnancy rates the focus has shifted towards creating safer and milder treatment protocols with less chance of ovarian hyperstimulation[13] and less chance of multiple pregnancies by transfer of one single embryo[14], especially since the improvement of cryopreservation of surplus embryos has created fair chances of pregnancy after transfer of frozen-thawed embryos[15].
As a result of all the developments over the past decades, by now it is possible to retrieve oocytes in almost 95% of the couples, around 85% of treatment cycles result in transfer of carefully selected embryo(s) that appear to have undergone

the optimal morphological development [16]. Often surplus embryos can be cryopreserved and transfered in a later cycle. Although around 30% of all embryo transfers result in clinical pregnancy, ongoing pregnancy rates are around 25%[16]. This remains lower than the success rates after natural conception; 40% clinical pregnancies and 30% ongoing pregnancies [17]. Given these 'natural' success rates, there should still be some possibility for improvement of artificial success rates. As illustrated in this introduction, we have improved separate steps of IVF treatment; therefore it seems logical that the focus should now be shifted towards the process after the final step in IVF treatment.

After the embryo has been transferred, the embryo is out of reach for further influencing of the chance of implantation. It continues on a more 'natural' journey towards a natural process of implantation: embryo and endometrium begin their intrinsic dialogue through many different factors to establish embryo implantation by the processes of apposition, attachment and invasion of the endometrium [18].

The implantation process is complex and often regarded as the 'black box' of pregnancy. Unravelling the secrets of this 'black box' can be compared with understanding lost languages of ancient cultures: gradually we discover more bits and pieces that are involved in the implantation process, but we know that we might still have to find our Rosetta Stone, that will help us understand the process in total. Therefore, embryo implantation remains subject to research, as it is the final and most important step towards pregnancy. With all bits and pieces that are discovered, the possibility may arise to influence and improve the implantation process and as a consequence improve pregnancy rates in IVF-treatment.

The implantation process and the uterine environment have to be investigated from a small distance, because the embryo is out of reach and the implantation process cannot be monitored or investigated directly. But it is possible to investigate the circumstances under which the implantation process has to take place and to investigate intrinsic or applied factors that could influence or reflect the strength of the implantation process.

Aim of this thesis

In this thesis, we started to investigate factors involved in the implantation process, followed by the investigation of techniques and medication as contributing factors to the implantation process. The aim of this thesis was to answer to following questions:

- What is the value of a single measurement of human chorionic gonadotrophin (hCG) regarding the strength of embryo implantation and treatment outcome?
- Which patient or treatment related factors contribute to the difference in chance of ongoing pregnancy between singleton and multiple implantations after IVF?
- Does multiple implantation have a hereditary basis and could it therefore influence chances of multiple implantation after artificial multiple ovulation?
- Is the chance of implantation influenced by ultrasonographically monitoring of the embryo transfer or by the location where the transfer air bubbles are seen? And is the position of the transfer air bubbles influenced by immediate ambulation after embryo transfer?
- Is it possible to influence the chance of implantation or to improve the quality of the implantation by administration of low-dose aspirin during IVF treatment? And is uterine vascularisation improved in patients receiving low-dose aspirin during their IVF treatment?

Outline of the thesis

In **Chapter 2** we describe the optimisation of the cut off values of the hCG-levels after embryo transfer for discrimination between viable and non-viable pregnancies on a single serum hCG determination as a reflection of the strength of the implantation. In this retrospective study data of 204 positive serum pregnancy tests after IVF or ICSI were used to create cut-off values that were tested by another 487 positive serum pregnancy tests after IVF or ICSI.

In **Chapter 3** we investigate which patient or treatment related factors play a role in the continuation of pregnancy after 6 weeks gestational age and we compare those factors to the factors involved in determination of multiple embryo implantation in a retrospective data-analysis of 1148 singleton and 445 multiple pregnancies.

In **Chapter 4** we investigate whether multiple implantation is related to familiar history of twinning to estimate to what extent a positive family history of twinning can influence the chance of multiple implantation after double embryo transfer by means of a questionnaire study among 1593 patients with a clinical pregnancy after double embryo transfer in an IVF or ICSI treatment.

In **Chapter 5** we describe the value of ultrasonographically monitored embryo transfer compared to a more traditional technique based on uterine cavity depth in a study combining prospectively collected data of 367 ultrasonographically guided embryo transfers and retrospectively collected data of 363 embryo transfers based on previous ultrasonographic uterine length measurement.

In **Chapter 6** we analyse the location of the transfer air bubble after embryo transfer in relation to the clinical outcome of the IVF treatment in a prospective investigational study of 367 embryo transfers.

In **Chapter 7** we investigate whether there is difference in change of location of the transfer air bubbles between immediate ambulation after embryo transfer and bedrest after transfer in a prospective observational study of 120 embryo transfers.

In **Chapter 8** we investigate the effect of low-dose aspirin administration throughout the IVF treatment on pregnancy rates and Pulsatility Index (PI) of the uterine arteries in a prospective double blind placebo controlled randomised trial of 169 IVF and ICSI treatments.

In **Chapter 9** we investigate the outcome and pregnancy complications of the patients with ongoing pregnancy in a follow-up study by means of questionnaires and analysis of hospital records of 54 patients with an ongoing pregnancy in the prospective double blind placebo controlled randomised aspirin trial.

In **Chapter 10** we summarize and discuss the results of the studies presented in this thesis.

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2

Optimizing hCG cut-off values: a single determination on day 14 or 15 is sufficient for a reliable prediction of pregnancy outcome.

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Abstract

Objective: Optimizing the cut-off level for a single serum hCG determination around day 15 after oocyte retrieval or ovulation.

Study design: Retrospective data analysis.

Results: 204 hCG samples > 5 IU/L between March and October 1999 taken on day 14, 15 or 16 after oocyte retrieval in 204 patients undergoing IVF or ICSI were analyzed. ROC-curves and optimal cut-off levels to discriminate between viable and non-viable pregnancies were calculated for each day separately. Cut-off levels were found at 76, 142 and 223 IU/L for day 14, 15 and 16 respectively and were verified by 487 hCG samples >5 IU/L taken between January 2000 and June 2004.

Conclusions: A single serum hCG determination on day 14 or 15 is sufficient to discriminate viable pregnancies accurately from non-viable pregnancies.

Introduction

In 1927, Aschheim and Zondek found a substance in the urine of pregnant women, currently known as human Chorionic Gonadotropin (hCG) [1]. HCG is a glycoprotein and is composed of two distinct non-covalently linked subunits: the α- and β-subunits. HCG is mainly synthesized by the trophoblast[2] and both urinary and serum analysis are used for pregnancy testing[3]. Viable pregnancies follow a rapid logarithmic rise in serum hCG in a distinct pattern the first 45 days after conception with a wide range [2,4,5].

Patients undergoing assisted reproductive techniques (ART) like in vitro fertilization (IVF) or intracytoplasmatic sperm injection (ICSI) are a group of patients, in which the date of ovulation is known exactly; an injection of hCG is given to prepare for oocyte retrieval. This group of patients gives us the opportunity to study the discriminative value of early serum hCG levels between viable and non-viable pregnancies. Especially these patients are very anxious to hear the prognosis and thus will benefit most from accurate early cut-off levels. In our clinic the first hCG sample is taken around day 15 (14-16). Consecutive samples are taken at a weekly interval until values either rise above 1000 IU/L or decrease below 5 IU/L.

A number of authors have analyzed hCG values for cut-off values to discriminate viable pregnancies (that will develop into ongoing pregnancies after a positive test) from non viable pregnancies[6-20]. Most of these results are not generally applicable for daily practice, because analyses were done on various days post oocyte retrieval [7;13;15;17-19], data were pooled [6;8;9;12] or the day of sampling was related to the day of embryo transfer, which thus could vary from 2 to 5 days after oocyte retrieval [10;11;14;16] and, finally, in several studies the cut-off value found had a rather low predictive value[8-10;13;17]. Given these shortcomings we aimed to demonstrate that it can be possible to apply a meticulously timed single hCG estimate for accurate prediction of assisted reproduction pregnancy outcome.

Materials & methods

All IVF and ICSI-patients treated at the IVF-center of the VU University medical center with a positive pregnancy test between March 1999 and October 1999 were included in this retrospective study (group A). Patients enrolled in this study became pregnant after treatment with in vitro fertilization or intracytoplasmatic sperm injection. In our clinic it is standard to perform hCG sampling on day 15 after oocyte retrieval. If day 15 is a Saturday, patients are invited to come on day 14. If it is a Sunday, patients will come on day 16. All patients with a first serum hCG-level > 5 IU/L taken on day 14, 15 or 16 after oocyte retrieval were included. Extreme values were defined as mean plus or minus 3 SD and excluded from further analysis [21].

All serum hCG samples were measured using the ACS: 180 immunometrical assay (Bayer AG, Leverkusen, Germany). The intra-assay variation was 2-5% and the interassay variation 5-8%.

An ongoing pregnancy was defined as an intrauterine pregnancy with adequate fetal development and cardiac activity 10 weeks after oocyte retrieval.

To verify the cut-off levels found in group A we prospectively predicted pregnancy outcome for all IVF- and ICSI-patients with a first serum hCG-level >5 IU/L, taken on day 14, 15 or 16 after oocyte retrieval, between January 2000 and June 2004 (group B). These samples were also analysed with the ACS: 180 immunometrical assay (Bayer AG, Leverkusen, Germany).

The study was approved by the institutional review board of the Department of Obstetrics & gynaecology of the VU University medical center.

Statistical analysis

Continuous data were compared with a two sample t-test using SPSS 11.0 (SPSS, Illinois, USA). To determine the cut-off value a receiver operator curve (ROC-curve) was constructed with the use of MedCalc statistical software.

Results

Between March and October 1999, 204 patients had a positive first blood sample (hCG >5 IU/L) taken on day 14, 15 or 16 after oocyte retrieval (group A), between January 2000 and June 2004 there were 487 patients with a first hCG >5 IU/L (group B). Baseline characteristics are shown in Table I.

Table I
Baseline characteristics of the patients.
Group A: March-October 1999, Group B: January 2000-June 2004

Patient characteristics	Group A	Group B	P
Age (y)	35,1	34,4	0.023
Duration of infertility (y)	5,4	5,1	NS
Diagnosis			
Tuba factor	54(27%)	130(26,7%)	NS
Endometriosis	11(5,5%)	35(7,2%)	NS
Male factor	81(40,5%)	232(47,6%)	NS
Hormonal	5(2,5%)	11(2,3%)	NS
Idiopathic	42(21%)	77(15,8%)	NS
Cervix factor	3(1,5%)	1(0,2%)	NS
Failed refertilisation	2(1%)	1(0,2%)	NS
Immunologic	2(1%)	0	NS

NS= not significant

In group A four extreme values were excluded (1 day 14 value, 2 day 15 values and 1 day 16 value), in group B there were no exclusions. In group A, the remaining 200 values resulted in 108 (54%) ongoing pregnancies, 32 (16%) spontaneous abortions, 4 (2%) ectopic pregnancies and 56 (28%) biochemical pregnancies. Of the ongoing pregnancies there were 86 singletons, 21 twins and 1 triplet.

The average hCG-value of ongoing pregnancies was significantly higher than that of not ongoing pregnancies on all 3 days. Only on day 15 after oocyte retrieval the average hCG value for singleton pregnancies was significantly lower than that for multiple pregnancies (Table II).

Table II
Group A: Average hCG values (IU/L) for ongoing, not ongoing, singleton and multiple pregnancies for day 14, 15 and 16 after oocyte retrieval.

	Ongoing	Not ongoing	P	Singleton	Multiple	P
Day 14	190	33	<0,0001	179	254	NS
Day 15	249	58	<0,0001	216	283	0,006
Day 16	358	135	0,001	325	474	NS

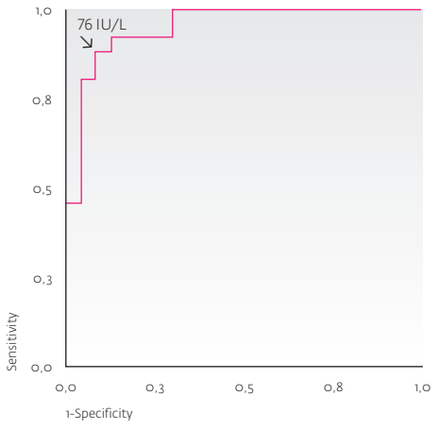
Using receiver operator curves (ROC-curves) the optimal cut-off values were calculated for each day separately. For samples taken on day 14 after oocyte retrieval the cut-off for an ongoing pregnancy was 76 IU/L (sensitivity 91,2%, specificity 91,3%, positive predictive value 93,9%, negative predictive value 87,5%), for samples taken on day 15 it was 142 IU/L (sensitivity 84,8%, specificity 94,7%, positive predictive value 94,9%, negative predictive value 84,4%) and for samples taken on day 16 it was 223 IU/L (sensitivity 72,2%, specificity 83,3%, positive predictive value 86,7%, negative predictive value 66,7%)(Figure 1). All ectopic pregnancies had hCG values below the cut-off values.
In group B, 89 patients had a first blood sample taken on day 14, 311 patients on day 15 and 87 patients on day 16 after oocyte retrieval. Using the cut-off levels found in group A, for each patient in group B the pregnancy outcome was predicted. From day 14 values, 60 out of 60 (100%) ongoing and 24 out of 29 (82,8%) not ongoing pregnancies were predicted correctly. From day 15 values, 188 out of 195 (96,4%) ongoing and 100 out of 116 (86,2%)not ongoing pregnancies were predicted correctly. From day 16 values, 42 out of 66 (63,6%) ongoing and 20 out of 21 (95,2%) not ongoing pregnancies were predicted correctly (Table III).

Table III
Prediction of pregnancy outcome in group B.

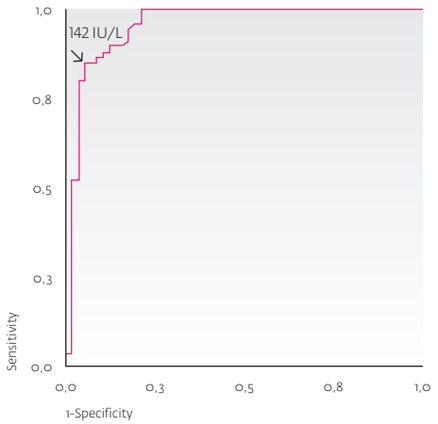
	Ongoing	Predicted	%	Not ongoing	Predicted	%
Day 14	60	60	100%	29	24	82,80%
Day 15	195	188	96,40%	116	100	86,20%
Day 16	66	42	63,60%	21	20	95,20%

Figure I
ROC- curves for day 14 (A), day 15 (B) and day 16 (C) after oocyte retrieval.

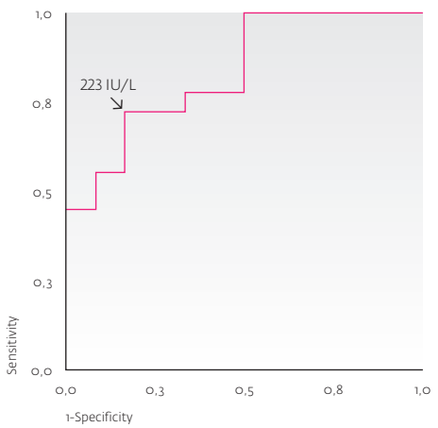
A: day 14



B: day 15



C: day 16



Discussion

Our data indicate that a single well-timed serum hCG estimate is highly predictive for pregnancy outcome. Using the cut-off levels found in this study, particularly measurements taken on day 14 and 15 after oocyte retrieval gave a very accurate prediction of both ongoing and not ongoing pregnancies. Day 16 measurements only gave a good prediction of not ongoing pregnancies, although it should be taken into account that the day 16 cut-off level was calculated on a smaller group. A single well-timed hCG estimate seems just as effective as a number of more complex modes of hCG measurements with or without combined estimates of other hormones [4;5;22-35].

Since the detection level of the assay was 5 IU/L, in daily practice we work with values from 5 IU/L onwards. Therefore, all values >5 IU/l were included in this analysis. Mannaerts et al[36] showed that on day 8 after Pregnyl administration hCG activity was <10% and serum levels had dropped to 10 IU/L. When serum samples are tested for hCG on day 15 after oocyte retrieval (day 17 after the Pregnyl injection), all values >5 IU/L can be regarded as the result of an invading trophoblast. Other studies[3;17] showed that even very low values (5-25 IU/L) can be associated with ongoing pregnancies.

If pregnancy occurs, hCG might be traceable in the maternal serum as early as day 8 after ovulation, but it should be present from day 12 onwards[37]. Taking the doubling time into consideration (1,3-1,89 days [5;37;38]) a sample taken on day 14 or 15 will give a good idea of the strength of implantation, because at that time several doublings of hCG values must have occurred in case of a good implantation. Only then values around the cut-off or higher are reached. Postponing the pregnancy test makes it less sensitive, because the range of values will be wider. Apparently a single hCG-sample early in pregnancy can tell us much about the further course of a pregnancy: an adequate serum hCG is a first sign of successful implantation [4;13;14]. Alahakoon et al[39] showed, that repeated measurements did not contribute further to the predictive value of a single measurement. A repeated measurement can provide useful extra information, when the first measurement is under the cut-off level[21].

In our clinic it appeared that the ideal cut-off was 76 IU/L for day 14 and 142 IU/L for day 15 after oocyte retrieval. These absolute values may not count for other clinics, due to a number of possible differences in hormone estimates between various laboratories. But the timing of this first hCG blood sample is applicable for every clinic.

Although the average hCG levels for multiple pregnancies were higher than the average for singleton pregnancies on all three days of sampling, this difference was only significant on day 15. An explanation for this finding may be, that the majority of our samples were taken on day 15 and therefore the number of

samples taken on days 14 and 16 were not sufficient to reach significance. Since other studies have shown significant higher hCG-levels for multiple pregnancies on various days after oocyte retrieval [8;15-17;19;20;40], it is likely that significance will be reached in larger groups.

Couples dependent on assisted reproduction for their subfertility usually have been confronted with many disappointments before entering an ART-program. Accurate cut-off levels for hCG values can give them more certainty early in treatment. We believe that with a single hCG sample taken on either day 14 or 15 after oocyte retrieval with an accurate cut-off level, much disappointment will be prevented. Values above the cut-off can be followed by a transvaginal ultrasound on day 30, whereas values below the cut-off should be followed by consecutive measurements.

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3

Factors determining early pregnancy loss in singleton and multiple implantations

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Abstract

Background: The incidence of first trimester pregnancy loss is much lower in IVF twin pregnancies than in IVF singleton pregnancies. The objective of this study was to determine, which embryonic and maternal factors contribute to this finding.

Methods: Retrospective data analysis of the outcome of 1593 pregnancies after day 3 double embryo transfer (DET) after IVF- or ICSI- treatment.

Results: Of 1148 single implantations at 6 weeks 936 (81.5%) were ongoing pregnancies. Of 445 multiple implantations at 6 weeks 354 (79.6%) were ongoing multiple pregnancies, 80 (17.9%) were ongoing singleton pregnancies and 11 (2.5%) ended in a spontaneous abortion. Total pregnancy loss was 18.5% and 2.5% ($p<0.001$) in singleton and twin gestations respectively. Loss per gestational sac was 18.5% and 11.46% ($p<0.001$) respectively. Determinants contributing to continuation of gestation beyond 6 weeks were: young maternal age, possibility to cryopreserve embryos and short GnRH agonist flare up stimulation protocol. Whereas factors promoting multiple implantation at 6 weeks of gestation were: young maternal age, high cumulative embryo score (CES), male infertility, long stimulation protocol and thick endometrium.

Conclusions: Although multiple implantation at 6 weeks is predominantly determined by (morphological) embryo quality, continuation of pregnancy beyond 6 weeks becomes more dependent on the combination of genetic and developmental potential of the embryo(s) and an optimal uterine milieu.

Introduction

The incidence of spontaneous abortion is the highest in the first trimester of pregnancy. Recent IVF studies showed a much lower incidence of pregnancy loss in pregnancies with twin nidation [1-3]. Remarkably enough, this finding does not only account for the incidence of total pregnancy loss: it was found, that the incidence of loss per gestational sac in multiple pregnancies is also much lower compared to singleton pregnancies [1].
It was suggested, that embryos of twin pregnancies come from a better cohort and have better intrinsic potential [1]. Van Royen [4] showed, that top quality embryos do have a better chance of implantation, but the question remains, whether these embryos also have better chances of continuation of pregnancy. La Sala [5] hypothesized, that the embryonic potential for early development is not the same for twins and singletons. Finally, Zegers-Hochschild [2] postulated that women with multiple gestation represent highly fertile individuals. Nevertheless, the studies published so far assumed rather than proved the dominant role of the embryo quality with regard to the observed better maintenance of pregnancy in case of multiple implantation with IVF. In the current study, we aimed to differentiate between embryonic quality and maternal factors contributing to the chance of multiple implantation and subsequent pregnancy loss. For this purpose, we performed logistic regression analyses of routinely registered variables with emphasis on careful quality classification of the embryos.

Materials & methods

We reviewed records of all IVF/ICSI patients treated in our centre between 1 January 2000 and 31 December 2004 and included all patients who met the following inclusion criteria: fresh IVF- or ICSI- treatment cycle, double embryo transfer (DET) on day 3 after oocyte retrieval, positive serum pregnancy test on day 14-16 after oocyte retrieval, presence of one or more intrauterine gestational sac(s) on ultrasound at 6 weeks of gestation. Only one treatment cycle per patient was included. If patients had more cycles resulting in pregnancy within the study period, the first cycle was included. Pregnancies after cryopreservation, ectopic pregnancies and anembryonic pregnancies were excluded. Data regarding patient characteristics, treatment, embryo development and treatment outcome were collected.

Of all patients the following information was collected for analysis: maternal age, duration of child wish, duration of infertility, previous pregnancy (yes/no), indication for treatment, type of treatment (IVF/ICSI), treatment protocol (short/long), duration of stimulation, endometrial thickness, level of estradiol (E_2), number of follicles, number of oocytes, number of fertilised oocytes, number of embryos available for transfer, possibility of cryopreservation, number of embryos available for cryopreservation, morphological embryo score, pregnancy monitoring data.

Embryo development was evaluated shortly before embryo transfer, and the best two embryos were selected for transfer. Each embryo was scored for morphology (grade 1 to 4) according to its symmetry and the extent of fragmentation of the blastomeres [6;7], an optimal quality embryo received score 1.

The Cumulative Embryo Score (CES) was calculated according to the example of Steer [8], adjusted for day 3 embryos. For calculation of the CES an embryo with grade 1 morphology received 4 points, grade 2 received 3 points, etc. Basically, the CES for day 2 embryos is obtained by multiplication of the morphology score with the number of blastomeres and summarizing the scores of all embryos transfered per patient. Seven or more blastomeres on day 3 is considered one of the criteria for a top quality embryo [4]. For day 3 embryos the CES was adjusted as follows: embryos with ≥ 7 blastomeres received 3 points, embryos with 5 or 6 blastomeres received 2 points and embryos with ≤ 4 blastomeres received 1 point. In this study the CES was obtained by multiplication of the scores for morphology and number of blastomeres per embryo and summarising the scores of both embryos transfered per patient.

A pregnancy test was performed 14-16 days after oocyte retrieval. Positive tests were followed by transvaginal ultrasonographic monitoring at 6, 9 and 12 weeks of gestation. If cardiac activity could not be diagnosed at 6 weeks of gestation, ultrasonographic monitoring of the pregnancy was repeated after a

week. A clinical pregnancy was defined as a positive pregnancy test followed by intrauterine embryonic sac/parts at 6 weeks of gestation. An ongoing pregnancy was defined as an intrauterine pregnancy with one or two foetuses showing cardiac activity at 12 weeks of gestation. Spontaneous abortion was defined as intrauterine pregnancy with fetal cardiac activity at 6 weeks of gestation followed by fetal demise.

Statistical analysis

Statistical analyses were done by t-tests, χ^2-tests and binary logistic regression analysis with backward likelihood ratio. In the regression analysis the dependent variables were: number of implantations at 6 weeks (single/multiple) and loss of gestational sac before 12 weeks (yes/no). Two-sided $p < 0{,}05$ was considered statistically significant.

Results

Between 1 January 2000 and 31 December 2004, we performed 8552 'fresh' IVF/ICSI treatment cycles in our centre. A total of 6959 cycles was excluded for the following reasons: embryo transfer was not performed on day 3 after oocyte retrieval (2546 cycles), treatment did not result in pregnancy (3924 cycles), patients had more than one treatment cycle resulting in pregnancy during the study period (245 cycles), there was single- or triple- embryo transfer (115 cycles), there was no gestational sac on ultrasound at 6 weeks of gestation (106 cycles), pregnancy was ectopic (17 cycles), no embryo transfer had taken place (4 cycles) or data were incomplete (2 cycles). A total of 1593 treatment cycles with clinical pregnancy remained for analysis.

The average age of the patients was 33.7 years. There were 864 pregnancies resulting from IVF treatment and 729 from ICSI treatment. At 6 weeks of gestation, there were 1148 pregnancies with single implantation and 445 pregnancies with multiple implantation. Of the pregnancies with single implantation 936 (81.5%) were ongoing at 12 weeks. Of the pregnancies with multiple implantation 354 (79.5%) were still multiple pregnancies at 12 weeks, 80 (18%) spontaneously reduced to a singleton pregnancy and 11 (2.5%) ended in a complete spontaneous abortion (Table I). The ongoing pregnancy rate for singleton and multiple pregnancies was 81.5% and 97.5% (p<0.001) respectively, the risk of loss per implanted gestational sac was 18.5% and 11.46% (p<0.001) respectively (Table II).

Table I
Number of implantations at 6 weeks of gestation and the number of implantations with cardiac activity at 12 weeks of gestation.

Number of implantations at 6 weeks of gestation		Number of implantations with cardiac activity at 12 weeks of gestation		
	Total	0	1	2
1	1148	212	936	
2	445	11	80	354
Total	1593	223	1016	354

Table II
Number of single and multiple implantations and gestational sacs.

	Single implantation	Multiple implantation	P
N° of pregnancies at 6 weeks [a]	1148	445	
N° of pregnancies at 12 weeks [b]	936	434	
Percentage total loss	18.50%	2.50%	<0.001
N° of gestational sacs at 6 weeks	1148	890	
N° of gestational sacs at 12 weeks	936	788[c]	
Percentage loss per gestational sac	18.50%	11.46%	<0.001

[a] Pregnancy is defined when ultrasound reveals one or more implanted gestational sacs.
[b] Ongoing pregnancy is defined as an intrauterine pregnancy with adequate fetal development and cardiac activity at 12 weeks of gestation.
[c] 354 multiple pregnancies and 80 singleton pregnancies.

Univariate analysis between single and multiple implantations at 6 weeks gestation showed lower maternal age, more follicles at the time of oocyte retrieval, more fertilised oocytes, more embryos available for transfer, more embryos available for cryopreservation, thicker endometrium and higher cumulative embryo score for pregnancies with multiple implantation. These differences were statistically significant (Table III). All other variables were not significantly different.

Multivariate regression analysis (Table IV) for multiple implantation revealed that the variables young maternal age, presence of male infertility, long stimulation protocol, a thick endometrium and high cumulative embryo score were independently positively associated with multiple implantation at 6 weeks. All other variables were not significantly associated to multiple implantation.

Table III
Univariate analysis of routinely registered variables for singleton and multiple implantations.

	Single implantation	Multiple implantation	P
Maternal age (years)	33,86	33,26	0,004
N° of follicles	14,9	15,7	0,035
N° of fertilised oocytes	11,1	11,9	0,008
Embryos available for ET	7,6	8,1	0,018
Cryopreservation possible	21,30%	29,20%	0,001
Endometrial thickness	10,8	11,1	0,011
Cumulative embryo score	18,6	20	<0,001

Table IV
Multivariate regression analysis for multiple implantation at 6 weeks of gestation.

	Odds ratio (95% confidence interval)
Maternal age (years)	0.968 (0.938-0.999)
Male infertility	1.517 (1.109-2.076)
Long stimulation protocol	1.373 (1.08-1.745)
Endometrial thickness (in mm)	1.078 (1.046-1.11)
Embryo quality (CES)	1.073 (1.018-1.31)

Univariate analysis between pregnancies with and without loss of gestational sac(s) showed higher maternal age and a lower percentage of cycles with cryopreservation for pregnancies with loss of gestational sac(s). These differences were statistically significant (Table V). All other variables were not significantly different.
The multivariate regression analysis (Table VI) for pregnancy loss for all pregnancies revealed that the variables short stimulation protocol, possibility to cryopreserve embryos and young maternal age were independently positively associated with continuation of pregnancy. All other variables, including cumulative embryo score, were not significantly associated to multiple implantation.
Regression analysis for pregnancy loss for singletons showed exactly the same associated variables as regression analysis for pregnancy loss for all pregnancies, whereas regression analysis for pregnancy loss for multiples revealed only maternal age as an associated variable (Table VI).

Table V
Univariate analysis of routinely registered variables for pregnancies with and without embryonic loss.

	With pregnancy loss	Without pregnancy loss	P
Maternal age (years)	34,7	33,5	<0,001
Cryopreservation possible	18,20%	24,70%	0,015

Table VI
Multivariate regression analysis for embryonic loss[1] between 6 and 12 weeks of gestation.

	All pregnancies Odds ratio (95% confidence interval)	Singletons Odds ratio (95% confidence interval)	Multiples Odds ratio (95% confidence interval)
Maternal age (years)	1.107 (1.066-1.15)	1.094 (1.046-1.15)	1.124 (1.046-1.21)
Cryopreservation possible	0.641 (0.456-0.9)	0.587 (0.381-0.9)	0.662 (0.376-1.163)
Long stimulation protocol	1.557 (1.101-2.2)	1.485 (1.002-2.2)	1.772 (0.829-3.793)

[1] Embryonic loss is defined as intrauterine pregnancy with fetal cardiac activity at 6 weeks of gestation followed by fetal demise.

Univariate analysis between patients treated with short and long stimulation protocol showed that patients treated with the short protocol were older, had less follicles, less oocytes, less embryos available for transfer and less embryos available for cryopreservation, but they had higher total levels of estradiol and higher levels of estradiol per oocyte (Table VII). All other variables were not significantly different.

Table VII
Univariate analysis of routinely registered variables for patients treated with short and long stimulation protocol.

	Short protocol	Long protocol	P
Maternal age (years)	34,5	33,5	<0,001
Nº of follicles	12,5	15	<0,001
Nº of oocytes	10,3	13	<0,001
Embryos available for ET	6,7	8	<0,001
Embryos for cryopreservation[1]	4,9	6	0,002
Total estradiol	8343	7548	<0,001
Estradiol/oocyte	877	617	<0,001

[1] Average number of embryos available for cryopreservation was calculated among patients who did have embryos remaining after selection for transfer.

Discussion

Successful implantation and maintenance of implanted embryo(s) depend on the quality of the embryo, the quality of the implantation site and their interaction. Our work shows that the vanishing of one twin is a common feature of double nidation [9;10], but on the other hand it confirms the earlier observations by Tummers [1], that embryos survive better after double implantation. This remarkably better survival poses some urgent questions: Which of the aforementioned features of early pregnancy development predominate in determining the continuation of a pregnancy? Are these features the same as those that determine multiple implantation?
It has been suggested that in case of double implantation, continuation of pregnancy is more likely, because embryos were selected from a better cohort of embryos [1]. Our multiple logistic regression analysis indeed indicates good morphological embryo quality as the strongest factor determining the chance of double implantation. This is in agreement with studies characterizing top-quality embryos and studies comparing SET with DET [4;11;12]. These studies show, that the morphological embryo score plays a pivotal role in the chance of pregnancy and multiple implantation rates. These studies do not show whether this morphological quality is also essential for continuation of pregnancy.
From our analysis for chance of pregnancy loss we found younger maternal age to be the strongest contributing factor. Furthermore, the possibility of cryopreservation and the application of the short GnRH -agonist protocol contributed positively to continuation of pregnancy. Although embryo quality itself did not contribute significantly to the chance of pregnancy loss, we could ask ourselves to what extent the factors we found can be interpreted as parameters for embryo quality. For maternal age and the possibility to cryopreserve embryos, this could be the case.
There is strong correlation between increasing maternal age and the chance of pregnancy loss [13], which is commonly attributed to the increasing rate of aneuploidy [14]. On the other hand, it was found for both older and younger groups of patients that only 36% of the morphologically good embryos were chromosomally normal after fluorescence in-situ hybridisation (FISH) analysis of blastomeres [15-17]. An embryo that is morphologically optimal may have high potential of further development and subsequent implantation. But there still is a good possibility of chromosomal abnormality and therefore of spontaneous abortion, because approximately 50% of all first trimester abortions are associated with cytogenic abnormalities [18].
Embryos for cryopreservation are supernumerary and must fulfil very strict morphological characteristics. In our clinic, there must remain at least 3 embryos after the selection of the embryos for transfer, each of them consisting of at least

6 blastomeres with morphology score 1 or 2 (not more than 10% fragmentation). These embryos are re-examined one day later and, in case of appropriate further development, selected for cryopreservation. Given these strict criteria, the possibility to cryopreserve can be regarded as an indicator for good embryo quality. However, it also depends on the quantitative ovarian response during hormonal stimulation, and as such, it represents maternal characteristics.
To our surprise, we found that application of a short GnRH agonist protocol was also positively associated with continuation of pregnancy. In our clinic, we apply this as treatment of first choice, when patients are older than 38 years or when there has been a previous poor response [19]. Intuitively, we rather expected an adverse implication of this characteristic on the likelihood of ongoing pregnancy. Comparing patients treated with a short protocol to patients treated with a long protocol, we found that, at time of hCG injection, the total E_2 levels and the E_2 levels per retrieved oocyte were significantly higher (Table VII). Oestradiol is the dominant hormone during the follicular phase and an important link in endometrial proliferation by up-regulation of progesterone receptors, vascular epithelial growth factor (VEGF), insulin-like growth factor-I (IGF-I), heparin binding epidermal growth factor (HB-EGF) and L-selectin [20]. Therefore, we speculate, but can not prove, that because of to the 'flare up' of endogenous gonadotropin secretion in the short protocol, a more optimal endometrium develops, which may influence the quality of the implantation site.
From this, we hypothesize that maternal age and the possibility of cryopreservation reflect embryo quality in genetic and developmental potential respectively, while the type of stimulation protocol used reflects the potential of the implantation site to support a pregnancy.
With regression analysis for pregnancy loss for only singleton implantation, we found exactly the same associated factors as regression analysis for pregnancy loss for all pregnancies in more or less the same order of significance. The regression analysis for pregnancy loss for only multiple implantations showed maternal age as the sole associated factor. The stimulation protocol and the possibility of cryopreservation remained next in line, but were no longer significantly associated. This loss of significance may be caused by the smaller group of multiples in combination with the much lower prevalence of pregnancy loss in multiple pregnancies.
There are other hypotheses that may explain why only maternal age remains significant for pregnancy loss in multiple implantations. It can be speculated that, in case of double implantation, the embryos collaborate. Matorras [21] found the best mathematical model for prediction of pregnancy and multiple pregnancy to be a 'collaborative model' based on the hypothesis that implantation of one embryo facilitates the implantation of (an)other embryo(s). Through the embryonic-endometrial dialogue, specific endometrial molecules are activated [20],

optimising the uterine environment for implantation. On the basis of this collaborative model, one could hypothesize that, in double implantation, the environment is not only optimised for implantation of (an)other embryo(s) but also for survival of additional embryo(s). A second explanation may come from the increased placental mass in early multiple implantations [7;22]. Twin placentas produce more hCG and progesterone than singleton placentas. Important factors in implantation, such as glycodeline A and MUC-1, are under progesterone control [20]. Through higher progesterone production in twin pregnancies, a superior hormonal support of the uterine milieu is created [22]. Both hypotheses suggest that, in multiple pregnancies the uterine milieu is more optimal for continuation of pregnancy, leaving (maternal age dependent) genetic potential as the sole determinant for pregnancy loss.

In contrast to its important role in determining chance of multiple implantation, the cumulative embryo score did not further contribute to the chance of continuation of pregnancy up to 12 weeks of gestation. However, one should bear in mind that the cumulative embryo score contributes to the chance of multiple implantation and that multiple implantations have a better chance of survival through the first trimester. Therefore, when two good quality embryos are transfered, patients have a higher chance of multiple implantation and, once there is multiple implantation, have lower chance of pregnancy loss. Other factors associated with multiple implantation were consistent with previous publications [23;24].

The likelihood of multiple implantation and subsequent continuation of pregnancy in case of double embryo transfer is dependent on a combined action of the embryo and the implantation site. Our analysis of data from women who became pregnant after DET on day 3 after oocyte retrieval indicates that occurrence of multiple implantation is predominantly dependent on (morphological) embryo quality, but continuation of pregnancy is more dependent on the combination of genetic and developmental potential of the embryo(s) and an optimal uterine milieu.

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4

A family history of twinning in relation to multiple implantation.

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Abstract

Background: A familial basis for dizygotic twinning is known for multiple ovulation. However, for multiple implantation this remains unclear. In IVF/ICSI 'multiple ovulation' is artificially induced. If multiple implantation is not hereditary, incidences of twins in families of patients with single and multiple implantation after IVF/ICSI with double embryo transfer should be comparable.

Methods: A questionnaire study was conducted among patients with intra uterine pregnancy at 6 weeks of gestation, after IVF/ICSI treatment with double embryo transfer three days after oocyte retrieval.

Results: There were 940 patients who gave their informed consent. For women with single implantation (group A), the incidence of one and of multiple twins among the family was 27.2% and 15.5% respectively. For women with multiple implantation (group B), this was 29.5% and 17.8% respectively, p=0.424. The incidence of one and of multiple twins among first degree relatives was 10.6% and 1.1% in group A; for group B this was 8.7% and 1.9%, p=0.469. Multivariate regression analysis did also not reveal 'twins in family' or 'twins in first degree' as an associated variable for multiple implantation at 6 weeks.

Conclusions: Incidences of twins in families of patients with single implantation and patients with multiple implantation after IVF/ICSI are comparable. Our data do not support the concept that multiple implantation is hereditary.

Introduction

There is a familial basis for dizygotic twinning[1;2]. Mothers of dizygotic twins more often have twins among their siblings than mothers of singletons[3;4], and the risk of multiple pregnancy is increased in women reporting a history of multiple pregnancies in first degree relatives[5]. The precise genetic basis of this hereditary twinning has not yet been elucidated, but it is known that in women from families with hereditary twinning multiple follicle growth and multiple ovulation occurs[6;7].

Multiple ovulation must be followed by multiple fertilisation and multiple implantation to establish a multiple (dizygotic) pregnancy. It has been suggested that the endometrium of fertile women is very receptive and that this receptivity is a determinant for implantation and for multiple implantation[8]. This would imply that in women from families with twins the endometrium is also highly receptive for (multiple) embryo implantation. This would additionally contribute to a higher chance of multiple pregnancy. It is not known whether there is a familial basis for multiple implantation. In case of hereditary multiple implantation, one should also expect some occurrence of familial monozygotic twinning for which there are no strong indications in the literature.

IVF/ICSI can be regarded as a situation of artificially induced multiple ovulation and artificial multiple fertilisation. With assisted reproduction treatments, there are often two embryos transfered (double embryo transfer [DET]) after in vitro fertilisation or intra cytoplasmatic sperm injection. In a previous study, we demonstrated that good embryo quality, presence of supernumerous embryos allowing cryopreservation, thick endometrium and young maternal age are positive determinants for the chance of multiple implantation[9].

If multiple implantation is hereditary, we would expect a higher incidence of (any type of) twins in families of patients showing multiple implantation after IVF/ICSI with double embryo transfer compared to families of patients showing single implantation after IVF/ICSI with double embryo transfer.

In this study, we first investigated the incidences of twinning in families of women with multiple implantation after double embryo transfer and in families of women with single implantation after double embryo transfer. We then looked at whether a family history of twinning contributed to the chance of multiple implantation after double embryo transfer in IVF/ICSI-treatment, which would indicate some hereditary basis. If having twins in the family is a reflection of a higher implantation potential, this may also lead to lower chances of early pregnancy loss. Therefore, we also analysed whether spontaneous abortion rates between patients with and without family history of twins were different.

Materials & methods

Until the end of 2004, double embryo transfer was most common in our centre. All patients, who had an intact intrauterine pregnancy confirmed by ultrasonography at 6 weeks of gestation after double embryo transfer on the third day after oocyte retrieval in a 'fresh' IVF/ICSI treatment at our centre between 1 January 2000 and 31 December 2004, were eligible for this questionnaire study. Pregnancies after cryopreservation, ectopic pregnancies and anembryonic pregnancies were excluded. Data regarding patient characteristics, treatment, embryo development and treatment outcome had already been collected for previous analysis[9].

Patients who had a spontaneous abortion and patients with an unknown pregnancy outcome after 12 weeks of gestation, first received a letter announcing this study with the request to reply regarding whether we could send them further information about the study along with the informed consent form, the questionnaire and a return envelope. Patients who had an ongoing pregnancy directly received the information about the study along with the informed consent form, the questionnaire and a return envelope. Patients were requested to reply in all cases, whether they participated or refused to participate in the study. If we had not received a reply a month after the questionnaire had been sent, we contacted the patients to ask if they wanted to participate and if so, to ask them to return the informed consent form along with the answered questionnaire.

The questionnaire contained questions regarding family history of twinning[10] and on pregnancy outcome, smoking habits, use of alcohol and medication, all before and during treatment and pregnancy.

All data collected by means of the questionnaire were combined with routinely registered data as described previously[9]: maternal age, duration of infertility, previous pregnancy (yes/no), indication for treatment, type of treatment (IVF/ICSI), treatment protocol (short/long), data on treatment monitoring, number of embryos available for transfer, possibility of cryopreservation, number of embryos available for cryopreservation, morphological embryo score, pregnancy monitoring data. Data were analysed using *t*-test, x^2-test and multivariate regression analysis where appropriate.

The study was approved by the Institutional Review Board of the VU University medical centre.

Results

A total of 1593 questionnaires was sent to 1148 patients with single implantation (group A) and 445 patients with multiple implantation (group B). The response rate was 61.9%: 940 patients (59%) gave their informed consent (group A: 660 patients, group B: 280 patients), 48 patients (3%) refused participation. Baseline characteristics were not different between patients with and without family history of twins (Table I).

Table I
Baseline characteristics of patients with and without family history of twins.

	Twins in family	No twins in family	P
Maternal age (years)	33.7	33.6	0.649
Primary infertility (%)	62.5	59.7	0.411
Duration of infertility (years)	3.8	3.7	0.854
ICSI treatment (%)	49.2	45.2	0.245
First treatment (%)	46.7	46	0.656
Long GnRH protocol (%)	78.4	77.6	0.770
Indication for treatment			0.986
Tubal infertility (%)	18.6	18.9	
Male factor infertility (%)	51.2	52.2	
Idiopathic infertility (%)	14.9	14.7	
Other* (%)	15.3	14.2	

* Other implies: failed IUI, endometriosis, hormonal factor and cervical hostility.

There were 392 women (44.2%) had one or more sets of twins in her family: 266 (42.8%) in group A and 126 (47.5%) in group B (p=0.189). The incidences of one set of twins and of more than one set of twins in the family were 27.2% and 15.5%, respectively, in group A and 29.5% and 17.8%, respectively, in group B (p= 0.424). (Table II).

There were 101 women (10.7%) had one or more twins among first degree relatives: 73 (11.1%) in group A and 28 (10%) in group B. Incidences of one twin and multiple twins among first degree relatives were 10.6% and 1.1%, respectively, in group A and 8.7% and 1.9%, respectively, in group B (p=0.469). (Table II).

Table II
Incidences of twins in the family history for women with single implantation and women with multiple implantation.

	Single implantation	Multiple implantation	P
Family (total)			0.424
No twins	57.3%	52.7%	
1 twin	27.2%	29.5%	
>1 twin	15.5%	17.8%	
First degree relatives			0.469
No twins	88.3%	89.4%	
1 twin	10.6%	8.7%	
>1 twin	1.1%	1.9%	

There were 22 women who were part of a multiple pregnancy themselves: 6 of a monozygotic twin, 12 of a dizygotic twin and 4 of triplets. In group A the frequencies were 6 (0.9%), 6 (0.9%) and 4 (0.6%), respectively, and in group B 0 (0%), 6 (2.2%) and 0 (0%), respectively (p=0.086). There were 38 women who reported that they had had another pregnancy, other than the pregnancy selected in our study period, that was a multiple pregnancy: 33 (5%) in group A and 5 (1.8%) in group B, p=0.214.

There were 433 women (46.5%) who were smokers, 304 (46.6%) in group A and 129 (46.2%) in group B, p= 0.913. Of these smokers 59.8% reported to have quit smoking entirely, 31.4% had quit for a certain period (usually during treatment and/or during pregnancy) and 7.8% had never quit smoking. Again there were no statistically significant differences between the groups.

Alcohol use before treatment was also not different between the groups: 70.5% in group A and 67.9% in groups B, p= 0.462. Almost all patients used folic acid: 96.4% in group A and 99.3% in group B, p= 0.083. The majority of the women (87.6%) started the use of folic acid from the start of the IVF/ICSI-treatment; this was not different between the groups: 86.3% in group A and 87.5% in group B, p= 0.632.

Multivariate regression analysis for multiple implantation revealed that presence of a thick endometrium, application of a long GnRH-agonist stimulation protocol and high cumulative embryo score were variables associated with multiple implantation at 6 weeks (Table III). All other variables including 'twins in the family', 'smoking', 'preconceptional use of folic acid' and 'alcohol use' were not significantly associated with multiple implantation.

When multivariate regression analysis was repeated with 'twins with first degree pedigrees' instead of 'twins in family', analysis revealed male factor infertility, thick endometrium, long GnRH-agonist stimulation protocol and high cumulative embryo score as variables associated with multiple implantation at 6 weeks (Table IV). Otherwise, all other variables remained not significantly associated with multiple implantation.

Table III
Multivariate regression analysis for multiple implantation at 6 weeks of gestation (including variable 'twins in family').

	Odds ratio (95% confidence interval)
Endometrial thickness (mm)	1.086 (1.007-1.172)
Long stimulation protocol (no/yes)	1.676 (1.074-2.616)
Cumulative embryo score (CES)	1.091 (1.045-1.139)

Table IV
Multivariate regression analysis for multiple implantation at 6 weeks of gestation (including variable 'twins among first degree relatives').

	Odds ratio (95% confidence interval)
Indication for treatment: male factor infertility (no/yes)	1.940 (1.182-3.183)
Endometrial thickness (mm)	1.106 (1.026-1.192)
Long stimulation protocol (no/yes)	1.931 (1.227-3.039)
Cumulative embryo score	1.092 (1.046-1.140)

The spontaneous abortion rate for patients with a family history of twins and for patients without a family history of twins was 10.2% and 12.1%, respectively. The percentage loss per gestational sac was 11.2% and 16.1%, respectively. These differences were not statistically different. Multivariate analysis also revealed that 'twins in family' was not associated with pregnancy loss between 6 and 12 weeks of gestation. The only variable associated with pregnancy loss was advanced maternal age (Table V). When multivariate regression analysis was repeated with 'twins with first degree pedigrees' instead of 'twins in family', analysis revealed 'twins in first degree' was also not associated with pregnancy loss, but revealed that possibility of cryopreservation was a variable positively associated with continuation of pregnancy (Table VI).

Table V
Multivariate regression analysis for pregnancy loss between 6 and 12 weeks of gestation (including variable 'twins in family').

	Odds ratio (95% confidence interval)
Maternal age (years)	1.095 (1.035-1.159)

Table VI
Multivariate regression analysis for pregnancy loss between 6 and 12 weeks of gestation (including variable 'twins among first degree relatives').

	Odds ratio (95% confidence interval)
Cryopreservation possible (no/yes)	0.578 (0.350-0.954)
Maternal age (years)	1.116 (1.053-1.182)

Discussion

Dizygotic twinning is a biological process involving multiple ovulation, multiple fertilisation and subsequent multiple implantation. Previous studies have demonstrated that multiple ovulation, among other causes, has a familial basis [1;2], but it remains unknown whether the familial basis of dizygotic twinning additionally accounts for multiple implantation. Therefore, we investigated the incidence of twins in the families of women who became pregnant after IVF/ICSI-treatment with double embryo transfer, which can be considered as an artificial situation of multiple ovulation and multiple fertilisation.

Our study shows that there is no statistically significant difference in the incidence of twins in the family between patients who had multiple implantation after IVF/ICSI and those who showed single implantation after IVF/ICSI. Although in the general population the risk of multiple pregnancy is increased in women reporting a history of multiple pregnancies in first degree relatives[5], our findings make it unlikely that in IVF/ICSI-patients the chance of multiple implantation is influenced by their family history of twinning.

The multivariate regression analysis demonstrated once more that high cumulative embryo score, the application of a long stimulation protocol and thick endometrium are positively associated with multiple implantation. When analysis was repeated with 'twins with first degree pedigrees' instead of 'twins in family', it revealed that male factor infertility is also positively associated with the chance of multiple implantation.

It was not surprising to find high cumulative embryo score to be the strongest contributing factor. Studies on the characterization of top-quality embryos [11;12] and studies comparing SET and DET [13] have already demonstrated the important role of good embryo quality in chances of multiple pregnancy. These results indicate that morphologically optimal embryos have high potential for further development and implantation. Our findings underline the fact that the embryo quality plays an essential part in the chances of pregnancy and of multiple implantation.

The finding that application of a long stimulation protocol was associated with multiple implantation can be explained both as being an embryonic and a maternal characteristic. In our clinic, the long GnRH-agonist stimulation protocol is, in general, applied in younger patients and patients with previous good ovarian response [14] . It has been demonstrated that younger patients have higher chances of pregnancy [15] and higher chance of multiple implantation in case of double embryo transfer [16-18]. As such it can be regarded as a reflection of maternal characteristics. On the other hand, it can be a reflection of embryonic characteristics, since patients with good ovarian response to controlled ovarian hyper stimulation usually have around ten oocytes available for

IVF/ICSI, increasing the chances of multiple fertilisation and thereby creating the possibility of more optimal embryo selection. The possibility to select embryos and to cryopreserve supernumerary embryos can be regarded as an indicator for good embryo quality, since the transfered embryos are selected from a group of embryos with more or less equal morphology scores.

Thick endometrium can be regarded as a reflection of hormonal levels and endogenous maternal environment. From IVF-studies, it is known that for a good chance of pregnancy the endometrium needs to reach a certain thickness [19].

The positive association with multiple implantation that we found for male factor infertility can be explained by the fact that in these couples infertility is predominantly the result of low sperm quality. In most couples with severe male factor infertility the female spouse has no factor of infertility at all [20]. Therefore, these women have better chances of pregnancy and, in the case of double embryo transfer, higher chances of multiple pregnancy [21]. It remains unclear why this association was only found in the analysis with twins in first degree relatives.

Embryo implantation is an interplay between embryo and endometrium and biologically it seems logical to assume both play essential parts in this process. Matorras analysed several mathematical models for their predictive value for pregnancy and multiple implantation [8] . The 'collaborative model', based on the hypothesis that implantation of one embryo facilitates implantation of (an) other embryo(s) was found to be the most accurate in prediction of pregnancy and multiple implantation. Prediction models principally based on maternal aspects were clearly less accurate. Their results indicate, in line with our findings regarding the determinants for implantation, that the leading part in (multiple) implantation is played by the embryos.

Since previous studies demonstrated that high folate status [22], use of alcohol and smoking habits [5;23] may enhance chances of multiple pregnancies, our questionnaire also inquired after these compounds. Smoking and alcohol consumption have been shown to interfere with the estradiol feedback on FSH-production [23-25], resulting in an increased chance of multiple follicle growth and therefore multiple pregnancy. In our patient group, multiple follicle growth was induced by the IVF/ICSI treatment and we found no association with multiple implantation for smoking or alcohol consumption. One should keep in mind that behaviour like alcohol consumption is often underestimated [26] and that the majority of these patients quit smoking and alcohol consumption because of the treatment and their desire to become pregnant. But from these results, we hypothesize that smoking and alcohol consumption only contribute to an increased chance of multiple pregnancy because of an increased chance of multiple follicle growth.

A high folate status before pregnancy was recently found to increase the chance of twins [22]. Although there is still discussion on this subject, it is known that

the preimplantation embryo has an absolute need for folate for normal development [27]. High levels of endogenous folate before conception may enhance normal development of the preimplantation embryo(s) and therefore increase the chance of twin pregnancies [22]. In our study, almost all patients used supplementary folic acid, the majority already using it during treatment. This is a likely explanation why in our study the regression analysis did not reveal use of folic acid before conception as a variable associated with multiple implantation. Spontaneous abortion rates were not different between patients with and without a family history of twinning and 'twins in the family' was also not revealed as an associated factor for pregnancy loss in the first trimester by multivariate regression analysis. The variables found from the regression analysis were the same as in our previous publication [9]. Since the group of patients in this study is a selection of the group we analyzed for the previous publication the current outcome underlines the association of these variables with pregnancy loss.

In summary: dizygotic twinning is primarily based on multiple ovulation and subsequent multiple implantation. From the artificial model of multiple ovulation, we learned that the incidence of twins in the family and twins among first degree relatives is equal for women who have multiple implantation after double embryo transfer and women who have single implantation after double embryo transfer. Multivariate regression analysis demonstrates that 'twins in the family' or 'twins with first degree pedigrees' are not associated with multiple implantation at 6 weeks gestation. Therefore it is unlikely that multiple implantation itself is hereditary and therefore it seems that the familial basis for twinning is restricted to multiple ovulation.

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5

Ultrasonographic-guided embryo transfer does not enhance pregnancy rates compared with embryo transfer based on previous uterine length measurement

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Abstract

Study objective: To compare pregnancy rates (PRs) after ultrasoundguided-guided embryo transfer and embryo transfer based on ultrasonographic length measurement.

Design: Prospective intervention group combined with retrospective control group.

Setting: University fertility clinic.

Patient(s): Patients undergoing IVF and intracytoplasmatic sperm injection.

Intervention(s): Transabdominal ultrasonographic guidance at embryo transfer.

Main outcome measure(s): Pregnancy and implantation rates.

Result(s): In 367 ultrasonographic guided embryo transfers clinical pregnancy rate, ongoing pregnancy rate and implantation rate were 35.1%, 31.1% and 24.3% respectively. In 363 embryo transfers based on previous ultrasonographic length measurement the rates were 33.9%, 29.5% and 24.2% respectively. There were no statistical significant differences between the groups.

Conclusion(s): Ultrasonographic guidance does not show benefit in terms of pregnancy and implantation rates compared to previous ultrasonographic length measurement, another precise and atraumatic transfer technique.

Introduction

Embryo transfer is a very important, final step in IVF- and intracytoplasmatic sperm injection (ICSI) treatment. About 85% of all couples undergoing IVF or ICSI treatment reach this stage, but only one-third of them achieve ongoing pregnancy. The pregnancy rate following embryo transfer is dependent on multiple factors like embryo quality [1-3], endometrial receptivity [4] and the technique of the transfer itself. Uterine contractions [5;6], presence of blood on the catheter [7-10], bacterial contamination of the catheter [11], retained embryos [10], difficulty of transfer [5;12] and the type of the catheter [13-17] all influence the success rate of the IVF/ ICSI treatment.

The embryo transfer can be performed in three ways: [1] 'blind' (clinical touch); [2] based on information on the length of the uterus (obtained by previous ultrasonographic measurement or mock transfer); [3] guided by abdominal ultrasonography. Transabdominal ultrasonographic guidance during embryo transfer is one of the factors that may influence the outcome of the treatment [18-25]. During blind catheter insertion the catheter tip touches the fundus of the uterus inadvertently in 17.4% of the cases [26], which increases the frequency of uterine contractions [5] and may lead to a reduced pregnancy rate [6;27]. Several investigators found higher pregnancy rates after ultrasonographic guided embryo transfer compared to blind embryo transfer [19;21;23], while others did not find significantly higher pregnancy rates after ultrasonographic guided embryo transfer compared to blind embryo transfer [18;20;22]. Coroleu et al[28] found that the depth of embryo catheter influences pregnancy and implantation rates. The highest pregnancy and implantation rates were found when the tip of the catheter was placed 1.5-2.0 cm below the fundus.

Before introduction of ultrasound-guided embryo transfer in our clinic, we measured the length of the uterine cavity (fundus – external os) by transvaginal ultrasonography at the beginning of the treatment in all patients. Based on this measurement, at the embryo transfer the tip of the catheter was placed 1.5- 2.0 cm below the fundus, as described by Coroleu et al [28].

Most studies analyzing the effect of ultrasonographic guidance at embryo transfer compared this with clinical touch embryo transfer. To our best knowledge, there are no publications comparing ultrasound-guided embryo transfer to embryo transfer based on a previous ultrasonographic length measurement of the uterus. With this present study, we wanted to compare pregnancy rates of embryo transfer with ultrasonographic guidance to pregnancy rates of embryo transfer based on the ultrasonographic length measurement of the uterus.

Material & Methods

Since the use of ultrasound guidance was the subject of investigation the decision was made to include all consecutive patients undergoing a fresh embryo transfer after IVF or ICSI treatment during the period of November 2004 to February 2005. Patients were only included once, if patients had another transfer within this period the first transfer would be included. Patients undergoing embryo transfer after cryopreservation were excluded. Data were collected prospectively.

Stimulation protocol

Stimulation protocols and IVF procedures were carried out as previously described by Goverde [29] and Rooseboom [1] and their colleagues. In summary: patients ≤ 38 years or with previous good response in a IVF or ICSI treatment underwent controlled ovarian hyperstimulation with a 'long' protocol with GnRH-agonist (Decapeptyl; Ferring, Copenhagen, Denmark) and gonadotropins (Gonal F; Serono, Geneva, Switzerland or Puregon; Organon, Oss, the Netherlands).
In women > 38 years or with a previous poor response, a 'short' GnRH-agonist protocol was applied.
Ovarian response was monitored by vaginal ultrasonography and serum estradiol determinations. Human chorionic gonadotrophine (Pregnyl; Organon, Oss, the Netherlands) 10.000 IU s.c. was administered, when there was at least 1 follicle ≥ 18 mm and 3 or more follicles ≥16 mm. Ultrasound- directed oocyte retrieval was performed 36 hours later. Embryo transfer was generally executed on day 3 after oocyte retrieval. If only two or fewer embryos were available, the transfer was performed on day 2 after oocyte retrieval. In consultation between physician and the couple, a maximum of 2 embryos were transfered.

Embryo selection and embryo transfer

Directly before the transfer procedure, the embryo development and morphology score were determined and the best embryo(s) was/were selected. Each embryo was scored, 1 to 4, according to its symmetry and the extent of fragmentation of the blastomeres [13;30], an optimal quality embryo received score 1.
Patients were instructed to come with moderate bladder filling (last toilet visit 1.5-2 hours before embryo transfer). The patient was positioned in lithotomy position and the cervix was exposed using a bivalve speculum. The mucus in the cervical canal was removed with a cotton swab. The outer catheter of the Cook catheter (Cook Ireland Ltd, Limerick, Ireland) was positioned under guidance of abdominal ultrasonography. Then the inner catheter was loaded with the embryo(s) by the 'three-drop-technique', in which the embryo(s) is/are separated by an air bubble from a preceding and a following drop of medium [13], and was inserted through the outer catheter.

The inner catheter was positioned at 1.5-2.0 cm under ultrasonographic guidance. Then the embryo(s) were slowly released into the uterine cavity. The catheter was slowly removed and checked under a stereomicroscope to ensure there were no retained embryos. Embryo transfers were considered difficult when an obturator was required.
Embryo transfer was performed by one of six experienced physicians. Although ongoing pregnancy rates do vary among the individual physicians, these differences are not statistically significant [31].

Outcome

A serum pregnancy test was performed 14-16 days after oocyte retrieval. Pregnancies were monitored by transvaginal ultrasonography at 6, 9 and 12 weeks of gestational age. An ongoing pregnancy was defined as an intrauterine pregnancy with fetal cardiac activity 70 days after oocyte retrieval.

Control group

Results of the ultrasound-guided transfer were compared to a retrospective control group, consisting of patients who had a fresh embryo transfer after IVF/ICSI in the 4-month period (July-October 2004) before introduction of ultrasonographic guided embryo transfer. All consecutive patients were included; patients undergoing embryo transfer after cryopreservation were excluded, as well as patients that were already in the ultrasound- guidance group.
The length of the uterus was measured (from fundus to the external cervical os) at the start of the treatment cycle on a sagital view by transvaginal ultrasonography (Figure I).
Treatment procedure, embryo selection and embryo transfer were performed as described previously. The same type of catheter (Cook) was used. There had not been staff turnover or other changes in the procedures. The only difference between the groups was that in the control group the embryo transfer was performed without ultrasonographic guidance. In this control group the inner catheter was positioned at 1.5-2.0 cm based on a previous ultrasonographic length measurement of the uterus, therefore it was avoided touching the fundus.

The study was approved by the institutional review board of the Department of Obstetrics & gynaecology of the VU medical center, Amsterdam, the Netherlands.

Statistical analysis

Statistical analysis was performed using SPSS 11.5 software for Windows (SPSS Inc. Chicago, IL, USA). Data were compared by the unpaired *t*-test or x^2-analysis where appropriate.

Figure I.
Uterus length measurement (fundus-external cervical os).
A : transvaginal ultrasonographic view
B : schematic diagram

Figure IA

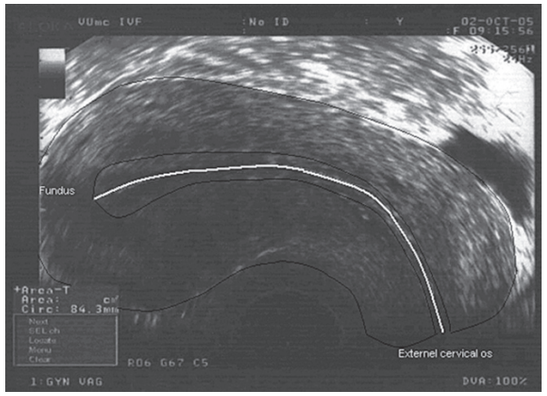
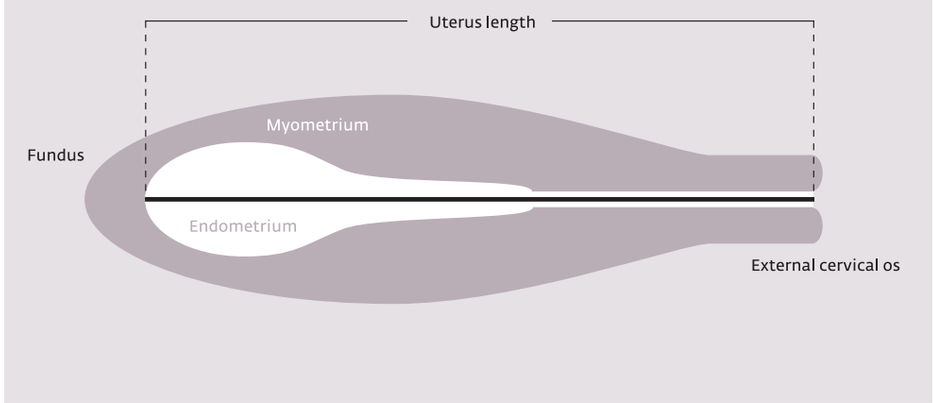


Figure IB



Results

A total of 367 embryo transfers in 367 patients were performed under ultrasonographic guidance. The clinical outcomes of these patients were compared to the clinical outcomes of the control group. The control group consisted of 363 embryo transfers in 363 patients based on a previous ultrasonographic length measurement of the uterus. Baseline characteristics are shown in Table I.
In the ultrasound-guided embryo transfer group, the mean age of the patients was 34.1 years. There were 207 transfers after IVF treatment and 160 transfers after ICSI treatment. In 95 transfers, one embryo was transfered (single embryo transfer [SET]) and in 272 transfers two embryos were transfered (double embryo transfer [DET]). An average of 1.74 embryos per patient was transfered. Of all transfers 129 (35.1%) resulted in clinical pregnancy, 114 (31.1%) pregnancies were ongoing.
In the control group, the mean age of the patients was 34.9 years. There were 192 transfers after IVF treatment and 171 transfers after ICSI treatment. In 82 transfers, one embryo was transfered and in 280 transfers two embryos were transfered. An average of 1.77 embryos per patient was transfered. Of all transfers 123 (33.9%) resulted in clinical pregnancy, 106 (29.2%) pregnancies were ongoing. The clinical and ongoing pregnancy rates, as well as the implantation rates were not significantly different between the groups (Table 1).

Table I.
Baseline characteristics and clinical outcomes of the US-guided embryo transfer group and the length measurement group.

	US-guided ET (n=367)	Length measurement ET (n=363)	P
Age (years)	34.1	34.9	0.009
ICSI treatment (%)	43.6	47	0.361
Primair infertility (%)	53.7	51.4	0.822
Duration of infertility (years)	3.7	3.4	0.083
First treatment (%)	40.1	35.6	0.219
IVF/ICSI attempt	2.2	2.4	0.073
Cause of infertility (%)			0.896
Tubal factor	16.6	19.3	
Male factor	48.2	48.9	
Endometriosis	6.5	6.4	
Idiopathic	23.7	21.3	
Other	4.9	4.2	
N° of embryos transfered	1.74	1.77	0.299
Difficult transfers (%)	6	6.6	0.724
N° of sacs at 6 weeks	155	156	0.433
Implantation rate (%)	24.30%	24.20%	0.564
Clinical pregnancy	129 (35.1%)	123 (33.9%)	0.739
Ongoing pregnancy	114 (31.1%)	106 (29.2%)	0.636

Note: ET= embryo transfer; US= ultrasonographic

Discussion

Our data indicate that pregnancy and implantation rates of embryo transfers based on a previous ultrasonographic length measurement of the uterus are comparable to pregnancy and implantation rates of transfers guided by abdominal ultrasonography. Because our control group was slightly older than the ultrasonographic guided group, we expected results in the ultrasonographic guided group to be better. Although the clinical pregnancy rate in the ultrasonographic guidance group is a little higher, this difference is still far from statistical significance.

As the embryo transfer is the final controlled step in IVF/ICSI treatment, it is important to transfer embryos in the best way possible. Strickler [32] was the first to describe the use of ultrasonographic guidance at embryo transfer as a possible improvement. Since then several studies have been published regarding the potential benefits of ultrasonographic guided embryo transfer.

In 2003, two meta-analyses were published: Buckett [25] analysed eight studies and Sallam [24] four studies. Both meta-analyses, however, compared clinical touch technique to ultrasonographic guided embryo transfer and found ultrasonographic guidance beneficial for pregnancy results. A few serious remarks concerning these meta-analyses should be made. In the meta-analysis of Buckett [25] four studies were quasi-randomised: the use of ultrasonography was determined either by alternate allocation [33] or the use of ultrasonography depended on availability of ultrasonography [18;34] or a specific room [35]. The other four were randomised trials [19-22], and were the same as analysed in the meta-analysis of Sallam and Sadek [24]. These four studies are different in some respects, that have been demonstrated to influence pregnancy results: the type of catheter used [13] and the depth of the transfer catheter [28]. It is also suggested, that a full bladder simplifies the embryo transfer procedure [20;36]. In each study patients received different instructions regarding having a full or empty bladder.

As mentioned, all of these previous studies compared results of ultrasound-guided embryo transfer to blind or clinical touch methods. During blind catheter insertion, the catheter tip touches the fundus of the uterus inadvertently in 17.4% of the cases [26], which increases the frequency of uterine contractions [5] and may lead to a reduced pregnancy rate [6;27]. The benefits of ultrasonographic guidance during embryo transfer may lie in the possibility to position the catheter more precisely and atraumatically.

From our data. it seems that the use of ultrasonographic guidance itself does not make the difference in pregnancy and implantation rates. As long as there is a previous length measurement of the uterus, it is possible to position the catheter at an optimum distance from the fundus in a precise and atraumatic way.

This study was carried out as a first analysis, whether the use of

ultrasonographic guidance at embryo transfer would help to improve the pregnancy and implantation rates compared to embryo transfer based on a previous ultrasonographic length measurement of the uterus. The study was carried out on our regular IVF/ICSI population; no selection was made. Although the preferred scientific approach would be a randomised controlled trial, given the very small differences found in our study a randomised trial would need very large numbers of patients to find significant differences. To find a difference of 5% in clinical pregnancy rate, with α= 0.05 and β= 0.80, the number of patients needed would be 1500 per group. Given our findings of only 1% difference in clinical pregnancy rate, for a prospective randomised controlled trial the number of patients needed would exceed 20000 per group. Such a trial would take years or should be performed as a multicenter study, with all additional disadvantages. Therefore, we decided not to frustrate ourselves on such an extensive project and were satisfied with the second best.

From this study, we conclude that when there is an accurate length measurement of the uterus, ultrasonographic guidance has no positive effect on pregnancy and implantation rates. The additional value of ultrasonographic guidance lies in other benefits, and for that reason only we have not abandoned this procedure. Abdominal ultrasonography can be of help in visualizing the catheter in the more difficult transfers, and the air bubbles can be documented. These aspects can be regarded as a confirmation of the transfer procedure. For both, patient and physician, ultrasonographic guidance adds to their confidence in the procedure: the physician receives visual feedback on what he or she is actually doing and the patient can see the air bubbles as a substitute for the embryo(s), that is/are transfered into her uterus. For improvement of pregnancy rates, ultrasonography has no value. To assure that the catheter is high enough without touching the fundus, a uterus length measurement is sufficient.

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6

The position of the transfer air bubbles after embryo transfer is related to pregnancy rate.

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Abstract

Study objective: The possibility to visualise the transfer air bubbles is one of the main benefits of ultrasound-guided embryo transfer. The objective of this study was to analyse the relation between the position of the air bubbles and pregnancy rates.

Design: Prospective data-analysis.

Setting: University fertility clinic.

Patient(s): IVF- and ICSI- patients.

Intervention(s): Transabdominal ultrasonographic guidance at embryo transfer.

Main outcome measure(s): Pregnancy rate, length endometrial plate, distance catheter to fundus, distance air bubbles to fundus.

Results: Analysis of 367 consecutive ultrasonographic guided embryo transfers following IVF- or ICSI-treatment. Both absolute and relative position of the air bubbles were significantly closer to the fundus in patients who became pregnant compared to patients who did not become pregnant, $p = 0.018$ and $p = 0.001$ respectively. When the relative position of the air bubbles was in the fundal half of the endometrial plate pregnancy rates were significantly higher compared to the lower half of the endometrial plate, 43.0% and 24.4% respectively, $p = 0.002$. Multiple regression analysis revealed the relative position as an independently associated determinant for pregnancy.

Conclusions: The position of the air bubbles after embryo transfer is related to pregnancy rate, the highest pregnancy rates are found when the air bubbles end up closer to the fundus.

Introduction

Pregnancy rates following embryo transfer in IVF/ICSI treatment are dependent on multiple factors like embryo quality [1;2], endometrial receptivity [3] and the technique of the transfer itself [4]. The embryo transfer is the final moment in IVF/ICSI that can be influenced by physicians. Therefore, there has been much interest in analysing and optimising several aspects of the embryo transfer. One of the main topics in recent studies is the use of transabdominal ultrasonographic guidance during the transfer procedure [5;6]. Although there is no consensus on the effect on pregnancy and implantation rates, there are other important advantages of performing the transfer under ultrasonographic guidance [7]. This technique offers the opportunity to visualise the transfer catheter, the air bubbles, the endometrial cavity and the aspect of the endometrium.

Under ultrasonographic guidance the catheter can be positioned very accurately. A number of studies analysed the relation between catheter position and pregnancy rates and found that the catheter position does influence pregnancy rates [8-10].

The transfer catheter is usually loaded using a 'three drop technique', in which the drop of medium containing the embryo(s) is separated from a preceding and a following drop of medium by an air bubble [11]. When standing up directly after embryo transfer, 94.1% of the air bubbles show no movement [12]. Of all embryos, that implant successfully, 81% does so in the area, where the air bubbles were initially seen at embryo transfer [13]. Therefore, the air bubbles can be regarded as an indication of the position of the embryos [14].

Surprisingly enough, there are only a few studies analysing the relation between the air bubble position and pregnancy rates [15-17]. The ones that did are contradictive and do not position the catheter at a standardised distance from the fundus. In our clinic, we perform embryo transfer at a standardised depth of the transfer catheter according to the findings of Coroleu et al [8]. In this study, we wanted to analyse the relation between pregnancy and implantation rates and the position of the air bubbles after positioning the tip of the inner catheter 1.5-2 cm from the fundus under ultrasonographic guidance.

Material & Methods

All consecutive patients undergoing a fresh embryo transfer after IVF or ICSI-treatment during the period November 2004 until February 2005 were included in this prospective study. Patients were only included once; if patients had another transfer within this period the first transfer was included. Patients undergoing embryo transfer after cryopreservation were excluded. Oocyte donation cycles were not included in this study.

Stimulation protocol

Stimulation protocols and IVF procedures were carried out as previously described by Roseboom [1] and Goverde [18]. In summary: patients ≤ 38 years or with previous good response in a IVF or ICSI treatment underwent controlled ovarian hyperstimulation with a 'long' protocol with GnRH-agonist (Decapeptyl, Ferring, Copenhagen, Denmark) and gonadotropins (Gonal F, Serono, Geneva, Switzerland or Puregon, Organon, Oss, the Netherlands). In women > 38 years or with a previous poor response a 'short' GnRH-agonist protocol was applied. Ovarian response was monitored by vaginal ultrasonographic and serum estradiol determinations. Human chorionic gonadotrophine (Pregnyl, Organon, Oss, the Netherlands) 10.000 IU s.c. was administered, when there was at least 1 follicle ≥ 18 mm and 3 or more follicles ≥ 16 mm. Ultrasonographic directed oocyte retrieval was performed 36 hours later.
Embryo transfer was generally executed on day 3 after oocyte retrieval. If only two or fewer embryos were available the transfer was performed on day 2 after oocyte retrieval. In consultation between physician and the couple a maximum of 2 embryos were transfered.

Embryo selection and embryo transfer

Directly before the transfer procedure, the embryo development and morphology score were determined and the best embryo(s) was/were selected. Each embryo was scored, 1 to 4, according to its symmetry and the extent of fragmentation of the blastomeres [11;19]. An optimal quality embryo received score 1.
Embryo transfer was performed by one of six experienced physicians. Although ongoing pregnancy rates do vary among the individual physicians, these differences are not statistically significant [20]. All embryo transfers were performed under ultrasonographic guidance. Ultrasonographic guidance was performed by one physician, who also performed all ultrasonographic measurements. Patients were instructed to come with moderate bladder filling (last lavatory visit 1.5-2 hours before embryo transfer).
The patient was positioned in lithotomy position and the cervix was exposed using a bivalve speculum. The mucus in the cervical canal was removed with a

cotton swab. The outer catheter of the Cook catheter (K-JETS-7019-SIVF/ Sydney IVF® Embryo Transfer Set, Cook Ireland Ltd, Limerick, Ireland) was positioned under guidance of abdominal ultrasonography. Then the inner catheter was loaded with the embryo(s) by a 'three-drop-technique' [11], in which the drop of medium containing the embryo(s) is separated from a preceding and a following drop of medium by a bubble of air, and inserted through the outer catheter. Both air bubble and droplet volumes do not exceed 10 µl.
The tip of the inner catheter was placed 1.5-2 cm from the fundal myometrium-endometrial interface as measured by ultrasound. Then the embryo(s) was/were slowly released into the uterine cavity, and the distance between fundal myometrium-endometrial interface and the transfer air bubbles was measured. The catheter was slowly removed and checked under a stereomicroscope to ensure that there were no retained embryos.
During the study we noticed that on abdominal ultrasonography in all patients the upper part of the endometrium was clearly thicker. This was also previously described by Prapas et al [21]. We called this the endometrial plate (Figure IA).
In all patients the following was measured (Figure IA & B): length of endometrial plate (distance A), distance between fundal myometrium-endometrial interface and the tip of the inner catheter (distance B), and distance between fundal myometrium-endometrial interface and air bubbles (distance C). The distance between the tip of the catheter and the transfer air bubbles (distance D) was calculated by subtracting distance C from distance B (Figure IB). The relative position of the air bubbles with regard to the length of the endometrial plate was calculated by dividing the distance between air bubble and fundal myometrium-endometrial interface by the length of the endometrial plate (CA-ratio). The relative position of the air bubbles was calculated in relation to the endometrial plate because this feature can be measured in most patients, whereas it is more difficult to measure the whole length of the uterus by abdominal ultrasonography.
Data were completed with information obtained from patient records.

The intra-observer variation was monitored using Bland-Altman plots [22]. Measurements on twelve patients were carried out three times using blinded procedures. All observed differences fell within the acceptability zone.

Outcome

A serum pregnancy test was performed 14-16 days after oocyte retrieval. Pregnancies were monitored by transvaginal ultrasonography at 6, 9 and 12 weeks gestational age. An ongoing pregnancy was defined as an intrauterine pregnancy with fetal cardiac activity 70 days after oocyte retrieval.

Statistical analysis

Statistical analysis was performed using SPSS 11.5 software for Windows (SPSS Inc. Chicago, IL, USA). Data were analysed using unpaired *t*-test or x^2-analysis and binary logistic regression analysis with backward likelihood ratio (dependent variable was pregnancy).

The study was approved by the institutional review board of the of the Department of Obstetrics & gynaecology of the VU university medical center.

Figure I
View of the transfer catheter and the air bubble at embryo transfer.
A: transabdominal ultrasonographic view
B: schematic diagram

Figure IA

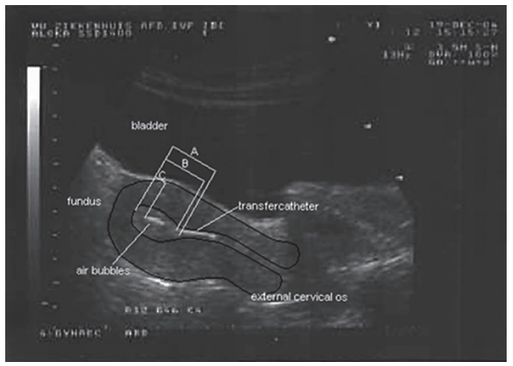
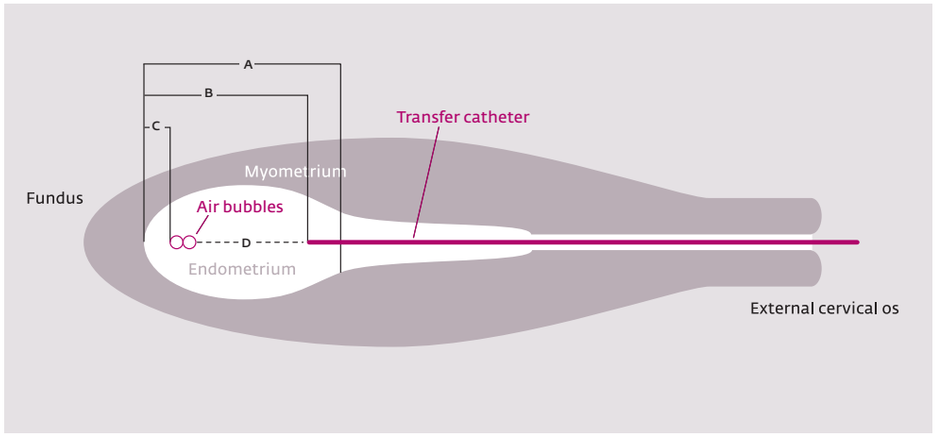


Figure IB



Results

A total of 367 embryo transfers in 367 patients was performed under ultrasonographic guidance. There were 207 transfers after IVF treatment and 160 transfers after ICSI treatment. In 95 transfers, one embryo was transfered (single embryo transfer [SET]) and in 272 transfers two embryos were transfered (double embryo transfer [DET]). Of all transfers 129 (35.1%) resulted in a clinical pregnancy, 114 (31.1%) pregnancies were ongoing.
There were few differences between the pregnant and non pregnant patients (Table I). The only statistical significant differences were found in the cause of infertility (higher percentage of male factor infertility within the group of patients that became pregnant), the possibility of cryopreservation, distance C and the CA-ratio. There was no difference is the number of difficult transfers (Table I), nor in the number of transfers with more than one problem at transfer (data not shown).

Table I
Baseline characteristics of the patients.

	Pregnant (N=129)	Not pregnant (N=238)	P
Age (years)	33.9	34.2	0.537
Cycle day 3 FSH (U/l)	6.24	6.96	0.037
Duration of infertility (years)	3.5	3.8	0.281
Primair infertility (%)	56.6	52.1	0.541
First attempt (%)	43.4	38.2	0.334
IVF/ICSI cycle number	2.1	2.3	0.191
ICSI treatment (%)	50.4	39.9	0.053
Cause of infertility (%)			0.033
Tubal factor(%)	14.7	17.6	0.473
Male factor (%)	55.8	44.1	0.032
Idiopathic (%)	24.8	23.1	0.715
Other[1] (%)	4.7	15.1	0.003
Long stimulation schedule (%)	72.1%	58.8%	0.014
Dosage FSH (per day)	175	209	0.001
Days of stimulation	11.5	11.3	0.637
Peak estradiol	7198	6858	0.515
N° of follicles	13.1	11.3	0.014
N° of oocytes	11	10.1	0.176
N° of embryos	7	6.2	0.069
Fertilisation rate (%)	68	63	0.029
N° of cryopreserved embryos	4.4	5.6	0.01
Cryopreservation possible (%)	36.4	21.4	0.002
Transfer on day 3 (%)	93	79	0.002
N° of embryos transfered	1.77	1.73	0.391
Problem at transfer (%)	19.4	25.6	0.177
Difficult transfers (%)	3.9	7.2	0.21
Blood stained catheter (%)	11.2	15.5	0.269
Mucus on catheter (%)	12.8	12.9	0.984
Retained embryos (%)	0.8	4.2	0.068
Endometrial thickness (mm)	10.8	10.7	0.492
Distance A (mm)	23.2	22.5	0.358
Distance B (mm)	17.5	17.9	0.461
Distance C (mm)	8.7	10.2	0.014
Distance D (mm)	8.7	7.7	0.065
CA-ratio	0.36	0.45	0.001

[1] Cause of infertility 'other': endometriosis, hormonal disorder, cervical hostility, immunological factors.

All air bubbles were recorded at the interface of the endometrial plate. The mean distance between the transfer bubble and fundal myometrium-endometrial interface (distance C) was smaller in the pregnant group (8.7 mm vs 10.2 mm, *p*=0.014). Following this, the relative position of the transfer bubble to the length of the endometrial plate (the CA-ratio) was lower in the pregnant group (0.36 vs 0.45, p=0.001). Regression analysis for pregnancy also revealed the CA-ratio as an independently associated determinant (Table II).

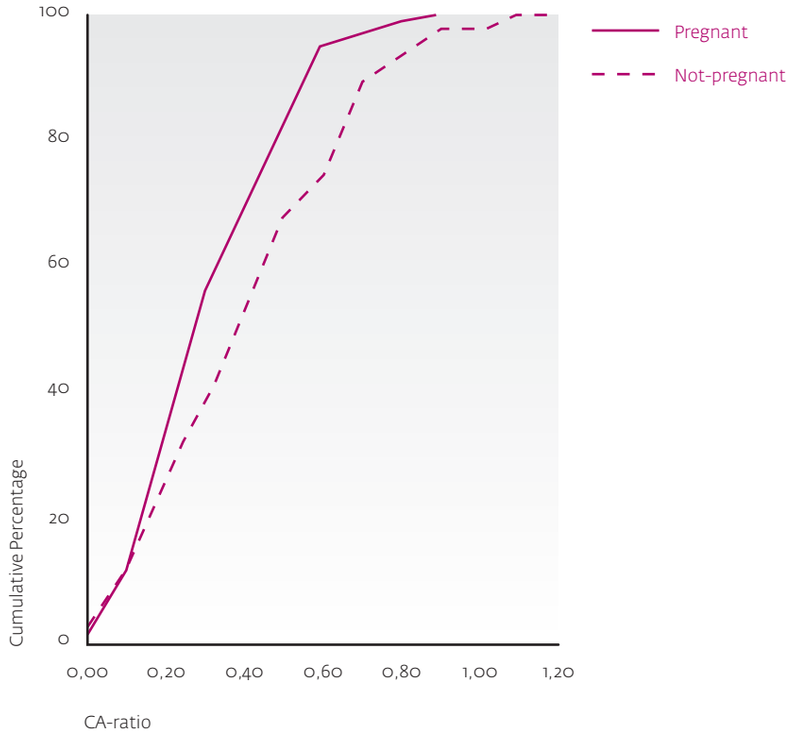
Table II

Multivariate regression analysis for pregnancy.

	Odds ratio (95% confidence interval)
Endometrial thickness	1.009 (0.874-1.166)
Treatment	0.919 (0.333-2.535)
Stimulation protocol	0.903 (0.458-1.781)
N° of embryos	1.139 (0.571-2.268)
Problem at transfer	0.829 (0.396-1.738)
Distance B	0.974 (0.909-1.045)
Age	0.971 (0.905-1.043)
Infertility primair/secundair	0.746 (0.414-1.346)
Day of transfer (day 2/ day3)	1.654 (0.607-4,510)
CA-ratio	0.24 (0.065-0.883)
Male factor	4.321 (1.139-16.388)
Tubal factor	5.136 (1.215-21.705)
Idiopathic infertility	6.306 (1.559-25.505)
Embryos for cryopreservation	2.571 (1.325-4.989)

Figure II displays the cumulative percentages of pregnant and not pregnant cases related to the CA-ratio, indicating that on average the air bubbles have a position closer to the fundal myometrium-endometrial interface in cases resulting in pregnancy. Pregnancy rates from cases where the position of the air bubbles was in the fundal half (CA-ratio ≤ 0.5) or in the lower half of the endometrial plate (CA-ratio > 0.5) were 43.9% and 24.4% respectively (p = 0.002). There was 1 case with retained embryos among the cases with a CA-ratio >1.

Figure II
Cumulative percentages of pregnant and not pregnant cycles related to the relative position of the air bubbles (CA-ratio).



Discussion

Our data indicate that the position of the transfer air bubbles after embryo transfer performed at standardized depth is related to pregnancy rates. For treatment cycles resulting in pregnancy the average position of the air bubbles, both absolute and relative, was closer to the fundal myometrium-endometrial interface. This was confirmed by multivariate regression analysis for pregnancy, in which the CA-ratio, the relative position of the air bubbles, was also revealed as an independently associated determinant.

Nowadays, many clinics perform embryo transfer under ultrasonographic guidance. One of the main advantages of the ultrasound-guided embryo transfer is the possibility to document the position of the transfer catheter and the air bubbles[7]. Several studies analysed the influence of the position of the catheter on pregnancy rates: Coroleu et al [8] showed, that catheter placement at 1.5 or 2.0 cm from the fundus was superior to catheter placement at 1.0 cm from the fundus. Pope et al [10] found, that pregnancy rates were higher, when the catheter was placed > 5mm from the fundus. For every additional millimeter of placement from the fundus, the odds of clinical pregnancy increased by 11%.

The transfer air bubbles are often regarded as an indicator for the position of the embryos: in the transfer catheter the embryos are sandwiched between the air bubbles. It is usually referred to as the 'double bubble sign' [14] or 'transfer flash' [16]. Because it was shown that 94.1% of the transfer air bubbles show no movement, even after standing up [12], and that 81% of the embryos, that implant successfully does so in the area where they were initially transferred [13], it was surprising that only a few studies analysed the relation between the location of the air bubbles and pregnancy rates [15-17].

In a small retrospective study analysing 23 pairs of pregnant and non pregnant cycles, Frankfurter et al [16] found that the relative position of the embryos (distance air bubble to fundus relative to the length of the endometrial stripe) was further from the fundus in pregnant cycles. This was followed by a prospective non randomized study [17] in which the transfers were directed to a certain part of the uterus. The final position of the embryos can not be predicted because it depends on multiple factors such as the syringe, the resistance of the plunger, the pressure used to press the plunger and possible intra uterine resistance, partly due to uterine contractions. The investigators did find better pregnancy rates when the transfer was directed to a lower part of the uterus, but unfortunately, in this study they did not analyse pregnancy rates to the relative position of the air bubbles. The lower pregnancy rates found in the group of transfers directed towards the uterine fundus may be a result of more traumatic transfers, resulting in more frequent uterine contractions, negatively affecting pregnancy rates.

In a study by Krampl et al [15] the position of the air bubbles themselves was analysed and the highest pregnancy rates were found, when the air bubbles ended up close to the fundus. Unfortunately, in this study the influence of the air bubble position on pregnancy rates was not a primary outcome, and was therefore not indicated precise.
Our study is the first to analyse the position of the air bubbles in relation to pregnancy rates after embryo transfer with a standardized depth of the transfer catheter. The depth of the transfer catheter was based on a study by Coroleu et al [8], that proved a positioning of the catheter at 1.5-2 cm from the fundus to be optimal. We found that both the absolute and the relative position of the air bubbles were closer to the fundus in pregnant cycles. The difference found in causes of infertility found between pregnant and non pregnant patients was in accordance with the findings of Roseboom et al [1], identifying the cause of infertility as a prognostic factor for probability of pregnancy.
An explanation for the relation between embryo position and pregnancy rates may be found in the difference in expression of factors involved in implantation. It is believed that the window of implantation is not only a temporal window but also a spatial window [3;23]: the expression of important factors in implantation such as leptin [24] and MUC-1 [25;26] differs throughout the endometrium. Therefore, it can be hypothesized that the expression of implantation factors in the fundal endometrium is more optimal for implantation. This is supported by the fact that in early pregnancies gestational sacs are mostly detected in the fundal area [13;27]. The position of the air bubble at embryo transfer was found to be relevant for pregnancy rates, but unfortunately, it is at present not possible to predict and/or control the position of the air bubbles: after positioning of the transfer catheter the final position of the air bubbles is dependent on the syringe, the resistance of the plunger, the pressure used to press the plunger and patient-related determinants as a possible intra uterine resistance. Therefore, we feel there is need for a more standardized method of embryo transfer, so the surplus value of exact positioning at embryo transfer can be analysed.

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7

Ultrasonographic evidence that bedrest after embryo transfer is useless.

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Abstract

Background: To demonstrate the effect of immediate ambulation after embryo transfer on the intrauterine location of the transfer content.

Methods: Prospective observational randomized controlled study.

Results: Fifty-seven patients (47.5%) had one air bubble at transfer and after 15 minutes, average change in position: group A (bedrest) 2.69 mm, group B (immediate ambulation) 2.00 mm (p=0.511). The distance from the fundus declined by 26% in group A and 15% in group B (p=0.229).
Twenty-eight patients (23.3%) had two air bubbles at transfer and after 15 minutes: average change in position of the first air bubble: group A 3.07 mm, group B 1.80 mm (p=0.282); average change in position of the second air bubble: group A 2.69 mm, group B 1.80 mm (p= 0.450). The distance from the fundus for the first air bubble declined 2% in group A and 18% in group B (p=0.593) and for the second air bubble 22% in group A and 15% in group B (p=0.711).

Conclusions: This study demonstrates that the transfer content is not affected by the immediate ambulation after transfer, illustrating why there is no rationale for bedrest after transfer.

Introduction

In the early days of IVF, it was believed that it was beneficial for the chance of pregnancy to keep patients in a horizontal position after the embryo transfer. To cut out the possible effect of gravity, embryo transfers have been performed with the patient positioned on her knees and elbows and it was very common to admit patients to the hospital for bedrest to prevent the embryos from 'falling out'.
A previous study showed that the duration of bedrest was not influential, by comparing pregnancy rates of patients who were admitted for bedrest for 1 hour or for 24 hours [1] and it was found that there was no difference in pregnancy rates. Given these findings, the whole idea of bedrest after transfer became rather questionable. A second study showed that indeed bedrest has no additional value for pregnancy rates in an IVF treatment comparing bedrest for one hour and immediate ambulation after embryo transfer [2].
Embryo transfer catheters are usually loaded by a 'three-drop-technique' [3], in which the drop of medium containing the embryo(s) is separated from a preceding and a following drop of medium by a bubble of air. With ultrasonographic guidance at embryo transfer it has become possible to visualise these air bubbles [4]. During embryo transfer the air bubbles often function as a marker for transfer content [3]. Because the initial position of the air bubble is related to the position of the early gestational sac [5], the air bubble position is generally regarded as marker for embryo position [6].
From the aforementioned studies [1;2] it can be concluded that there seems to be no difference in pregnancy rates between bedrest and immediate ambulation after transfer. Woolcott and Stanger showed that immediate ambulation shortly after embryo transfer does not influence the final position of embryo-associated air [7]. But there were no studies comparing air bubble location at transfer and a short period later, in patients remaining in horizontal position and immediately ambulated patients.
Using repeated ultrasonographic measurements of the air bubble location in patients remaining in horizontal position and immediately ambulated patients after the transfer we wanted to compare the change in position of the air bubbles between the two groups, as a final illustration that the location of the air bubbles is not influenced by immediate ambulation directly after the transfer.

Materials & methods

For 2 months, all consecutive patients undergoing a fresh embryo transfer after IVF or ICSI- treatment were included in this prospective observational study. Patients were informed about the study in advance and were asked for their consent on the day of embryo transfer. Patients were instructed to come with moderate bladder filling (last lavatory visit 1.5-2 hours before embryo transfer). Embryo transfer was performed as described previously [3]. In short: directly before the transfer procedure, the embryo development and morphology score were determined and the best embryo(s) was/were selected. All embryo transfers were performed under abdominal ultrasonographic guidance. With the patient in lithotomic position, the cervix was exposed using a bivalve speculum and the mucus in the cervical canal was removed with a cotton swab. Under abdominal ultrasonographic guidance the outer catheter of the Cook catheter (K-JETS-7019-SIVF/ Sydney IVF® Embryo Transfer Set; Cook Ireland Ltd, Limerick, Ireland) was positioned.

The inner catheter was loaded with the embryo(s) by the 'three-drop-technique', in which the drop of medium containing the embryo(s) is separated from a preceding and a following drop of medium by a bubble of air. After insertion through the outer catheter, the tip of the inner catheter was placed 1.5-2 cm from the fundal myometrium-endometrial interface as measured by ultrasound. Then the embryo(s) was/were gently released into the uterine cavity. The catheters were slowly removed and checked under a stereomicroscope to ensure that there were no retained embryos.

Randomisation was performed in blocks of 50 patients by an independent doctor using computerised tables that were modified by SPSS. Directly before the transfer, patients were allocated to group A (bedrest after transfer) or group B (immediate ambulation after transfer) using those computerised randomisation tables. After the catheter had been checked for retained embryos, patients in group A remained in horizontal position and in group B patients were immediately ambulated. In all patients, the embryo transfer was performed under abdominal ultrasonographic guidance. The location and diameter of the air bubbles (catheter content) were measured at the transfer. In all patients, these measurements were repeated 15 minutes after the embryo transfer. Patients were instructed not to visit the toilet before the measurement was repeated, just because of the ease of visualisation with a filled bladder. Ultrasonographic guidance at embryo transfer was performed by one physician (first author), who performed all ultrasonographic measurements, both at transfer and after 15 minutes. Measurements were performed on an Aloka 3500 device.

The 15 minute period was chosen, because in the past this had been the period patients would be lying down after transfer in our clinic and, more importantly, it would be unfriendly to have them wait longer with a full bladder. A previous study by Krampl [8] had shown that the air bubbles would be visible at least for an hour.

Because it was not known whether the location of the transfer content still changes after transfer, we hypothesized that in both groups the location of the transfer content will change some after transfer, but that there would be more change in the group with immediate ambulation. We figured that a 25% difference in change of location would be significant. With wanted α=0.05 and β=0.80 we needed to include 100 patients in this study.

Statistical analysis was performed using SPSS 15 software for Windows (SPSS Inc. Chicago, IL, USA). Data were analysed using unpaired *t*-test or x^2-analysis where appropriate.

The Institutional Review Board of the VU university medical center approved of this study.

Results

A total of 120 patients participated in this study. Sixty-four patients were allocated to group A (bedrest after embryo transfer) and 56 patients to group B (immediate ambulation after embryo transfer). In 18 patients, the first measurement was not possible, because the vision on ultrasound was compromised due to a high Body Mass Index (BMI), a fibroid uterus or an empty bladder, making visualisation very difficult. In 2 patients, the second measurement was not possible: one patient had visited the toilet between the transfer and the second measurement, in another patient the ultrasound image was unclear due to shading (Figure I). Baseline characteristics were not different between the two groups (Table I).

Figure I
Flowchart

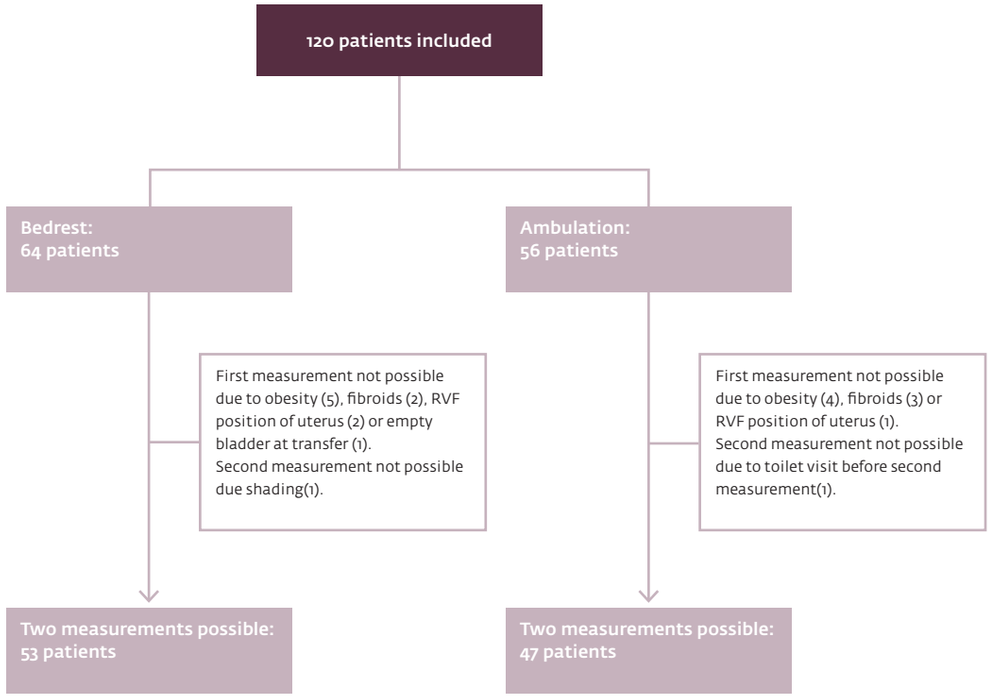

Table I
Baseline characteristics of the patients.

	Bedrest (Group A)	Ambulation (Group B)	P
Age (years)	34.91	34.88	0.971
ICSI (%)	52.80%	59.20%	0.518
Treatment cycle number	2.06	2.06	0.981
First cycle(%)	41.50%	57.40%	0.218
Primair infertility (%)	54.70%	54.30%	0.971
Duration of infertility (years)	2.98	3	0.957
Cause of infertility			0.427
tubal factor(%)	26.40%	14.30%	
male factor(%)	45.30%	49%	
idiopathic(%)	17%	18.40%	
other(%)*	11.30%	18.40%	
Long GnRH protocol (%)	55.80%	58.70%	0.770
FSH dosage (IE)	185	178	0.707
Duration of stimulation (days)	10.96	11.43	0.373
Endometrial thickness (mm)	10.33	10.74	0.392
Transfer on day 3 (%)	90.40%	89.80%	0.921

* Cause of infertility 'other':
endometriosis, hormonal disorders, cervical hostility and immunologic factors

Although there were always two air bubbles loaded into the catheter, after transfer both air bubbles were not visible on ultrasonography in all patients (Table II).

Table II
Number of air bubbles visible at embryo transfer and 15 minutes after transfer.

Number of air bubbles*			
Transfer	After 15 min	Bedrest (Group A)	Ambulation (Group B)
1	1	33	24
2	2	13	15
2	1	4	3
1	2	3	5

* No significant difference between the groups: p= 0.603.

In 57 patients (47.5%) one air bubble was visible both at transfer and after 15 minutes. Among these patients the average position of the air bubble had changed 2.69 mm in group A and 2.00 mm in group B (p= 0.511), in both groups towards the fundal myometrium-endometrial interface. The decline of the

distance between air bubble and fundal myometrium-endometrial interface was 26% in group A and 15% in group B (p=0.229). The diameter of the air bubble had declined 1.12 mm in group A and 1.25 mm in group B (p=0.742) (Table III).
In 28 patients (23.3%) two air bubbles were visible both at transfer and after 15 minutes. Among these patients the average position of the first air bubble (closest to the fundus) had changed 3.07 mm in group A and 1.80 mm in group B (p=0.282). The average position of the second air bubble had changed 2.69 mm in group A and 1.80 mm in group B (p= 0.450). Again, all changes were towards the fundal myometrium-endometrial interface. The decline of the distance between air bubble and fundal myometrium-endometrial interface for the first air bubble was 2% in group A and 18% in group B (p=0.593). The decline of the distance between air bubble and fundal myometrium-endometrial interface for the second air bubble was 22% in group A and 15% in group B (p=0.711). The diameter of the air bubble closest to the fundus had declined 0.84 mm in group A and 0.93 mm in group B (p=0.822) . The diameter of the second air bubble had declined 1.08 mm in group A and 0.93 mm in group B (p=0.750) (Table III).
In 7 patients (5.8%), two air bubbles were visible at transfer, but only one after 15 minutes. In this group one patient had an ectopic pregnancy. In 8 patients (6.6%), only one air bubble was visible at transfer that split into two separate air bubbles after 15 minutes. Since it is impossible to detect which bubble has appeared or disappeared making it is not possible to analyse any change in position; therefore these patients were left out of the analyses.

Table III
Change in position and diameter of the air bubbles[1]

	Bedrest (Group A)	Ambulation (Group B)	P
One air bubble visible			
Change in distance air bubble-fundus (mm)	2.69	2	0.511
Change in diameter of air bubble (mm)	-1.12	-1.25	0.742
Two air bubbles visible			
Change in distance air bubble 1 -fundus (mm) [2]	3.07	1.8	0.282
Change in diameter of air bubble 1 (mm) [2]	-0.84	-0.93	0.822
Change in distance air bubble 2 -fundus (mm) [3]	2.69	1.8	0.450
Change in diameter of air bubble 2 (mm) [3]	-1.08	-0.93	0.750

[1] Please note: changes in position of the air bubbles are in direction of the fundal myometrium-endometrial interface.
[2] Air bubble 1: the air bubble closest to the fundal myometrium-endometrial interface.
[3] Air bubble 2: the air bubble furthest from the fundal myometrium-endometrial interface.

Discussion

In this study, we investigated the changes in air bubble location between patients with bedrest and patients with immediate ambulation after embryo transfer. Our data show that the change in location of the air bubbles is not affected by immediate ambulation after transfer. With these results, we provide a likely illustration why there is no rationale for bedrest after embryo transfer. Since the transfer air bubbles are usually regarded as a marker for the transfer content, it is very likely that the location of the embryo(s) is also not affected by immediate ambulation after transfer.
Although we did find changes in the location of the transfer air bubbles, for both groups the direction of the change are similar and consistent with earlier reports on endometrial wave-like activity. Ijland [9] demonstrated that at the moment of embryo transfer the majority of the endometrial wave-like activity is directed from cervix to fundus. A possible function of this particular wave pattern is to restrict implantation of embryo(s) to the upper uterine cavity [10], where gestational sacs are mostly detected in early pregnancies [5;11]. Because the majority of the wave-like activity is directed towards the fundus, this explains the direction of the change in location of the air bubbles we found in our study. The reduction of the diameter of the air bubbles can be explained by the assumption that the air bubbles dissolve after a certain period.
When loaded into the catheter the embryos are located in between the air bubbles. No one knows whether this location persists after the transfer has been performed. In our study, we found that the air bubbles can not always both be visualized. If the embryos remain in between the air bubbles, it would be best to analyze only those patients in whom both air bubbles could be seen at both measurements. Since the change of location is best estimated if the number of visual air bubbles at transfer is equal to the number visualized after 15 minutes we analyzed those patients with equal numbers of visual air bubbles at both moments. The general assumption in tracking the air bubbles in embryo transfer [5;6;12] is that the effect on the air bubbles is the same as on the embryos. The 15 remaining cases were left out from analysis, because it is impossible to detect which bubble has appeared or disappeared, and therefore, it is not possible to analyse any change in position. For this, there can be several explanations: one air bubble at transfer becoming two may be due to their close initial position, and therefore the two bubbles were recognised as one, or it may have been due to working with two-dimensional ultrasonography. In those cases where two air bubbles at transfer became one, the reason for this may be fusion of the air bubbles or an extreme change in position of one of the air bubbles. It seems justifiable for these 15 patients were excluded from the data analyses, since with the disappearance or reappearance of air bubbles it is impossible to relate the

air bubbles from the first to the second measurement. Although without these patients the study does not reach the numbers that were calculated in the power analysis, these results still demonstrate that change in location of the air bubbles is not different whether patients had bedrest or were immediately ambulated; change in location was often even more in patients who had bedrest for 15 minutes.

The initial position of air bubbles at embryo transfer is regarded as a marker for the location of the embryos[6]. To the best of our knowledge it is not known for how long this association remains. The findings of Baba[5], who show that in 80% of the pregnancies after IVF the early gestational sac can be found in the area where the air bubbles were transfered initially may suggest that the association remains for a longer period of time. Recently, a case-report showed that the air bubble location does not need to be related to the position of the embryo[13]: in a patient with a bicornuate uterus with a very open angle the air bubble had been visualised at transfer in the left uterine horn at transfer of a single embryo. Three weeks later, the gestational sac was visualised in the other uterine horn. In this case report, the authors describe placing the tip of the catheter just beyond the internal os, because advancing beyond that point would have the catheter touch the uterine wall between the cavities. Perhaps the difference of the location of air bubble and gestational sac was a result of different angles of diversion after transfer in the direction of the uterine wall in between the two cavities of the bicornuate uterus. The fact remains that it is not known how long the location of the embryo remains related to the air bubbles nor whether the embryos may change location between transfer and first gestational sac measurement. We need to await further research projects since there is no absolute evidence at the moment and it will probably remain hypothetical unless we will be able and allowed by ethical committees to mark the embryos in a particular way.

In this study, we observed that the change in location of the air bubbles after embryo transfer is independent of immediate ambulation of the patient after transfer and that in both groups the average direction of the change in position was towards the fundal myometrium-endometrial interface. These findings illustrate previous findings why there is no rationale for bedrest after embryo transfer following IVF/ICSI.

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8

Low-dose aspirin in non-tubal IVF-patients with previous failed conception: a prospective randomised double blind placebo controlled trial.

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Candidate paper for the General Program Prize
ASRM annual meeting 2007

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Abstract

Objective: To analyse whether the administration of low-dose aspirin during IVF treatment improves the uterine blood flow and improves clinical and ongoing pregnancy rates for non-tubal IVF patients with previous failed conception.

Design: Prospective double-blind placebo-controlled trial. The trial is registered with the Dutch Trial Register and as an International Standard Randomised Clinical Trial, number ISRNCTM97507474.

Setting: University fertility clinic.

Patients: Non-tubal IVF patients with previous failed conception.

Intervention: Daily 100 mg aspirin or placebo throughout an IVF treatment with a long GnRH-agonist stimulation protocol.

Main outcome measures: Clinical and ongoing pregnancy rate, pulsatility index of the uterine artery.

Results: Of 169 patients, 84 were assigned to aspirin treatment and 85 to placebo treatment. In the aspirin group, 28 patients (35.4%) had an ongoing pregnancy and in the placebo group, 26 patients (31.0%) had an ongoing pregnancy, p=0.677. Multilevel analyses showed that the pulsatility index of the uterine artery was not affected by aspirin or placebo treatment.

Conclusions: Low-dose aspirin administration during IVF treatment does not improve pregnancy rates of non-tubal IVF patients with previous failed conception and it does not affect the arterial uterine blood flow.

Introduction

Aspirin is one of the best-known and most frequently used drugs and has found its way into various fields of medicine[1]. Aspirin is widely used in cardiology for prevention of cardiovascular diseases. In obstetrics, there is evidence that aspirin has beneficial effects in the prevention of pre-eclampsia and fetal growth restriction[2;3], and in women with recurrent abortion who are seropositive for antiphospholipid antibodies[4;5]. In the past decade, more evidence has become available that aspirin could also be beneficial in reproductive medicine.
Aspirin acelytates cyclo-oxygenase, through which it inhibits the synthesis of several prostaglandins and prostacyclins and thromboxane-A_2. When administered in low dosage, the effect of aspirin more selectively inhibits tromboxane-A_2, which induces platelet aggregation and vasoconstriction. Administration of low-dose aspirin therefore inhibits platelet aggregation and reduces vasoconstriction, resulting in an increase of blood flow. This improved blood flow would be beneficial for the build-up of the endometrium in the follicular phase[6].
Prostaglandins stimulate inflammatory cells and the release of interleukin, which produces inflammation and may therefore influence the process of implantation. Prostaglandins also stimulate uterine contraction, which also has an effect on implantation[7;8]. Altogether, the effect of aspirin is threefold: improvement of uterine blood flow, reduced inflammatory reaction and inhibition of uterine contractions. Each of these effects can help to improve the chance of successful implantation, which may contribute to higher pregnancy rates after IVF-treatment.
In the past decade several studies have analysed this potential benefit of aspirin in IVF-treatment, with various results[6;9-14]. In selected patient groups, promising results were found, whereas in unselected patient groups no benefits were found at all. Even though most of these studies were not sufficiently powered, it seemed that the beneficial effects of low-dose aspirin were restricted to selected patient groups.
Since aspirin is believed to improve the chance of implantation we hypothesize that patients with previous failed conception are a selected group of patients that may benefit from the effect of low-dose aspirin and therefore will have an improved chance of successful implantation and clinical pregnancy when treated with low-dose aspirin during their IVF-treatment.

Materials & methods

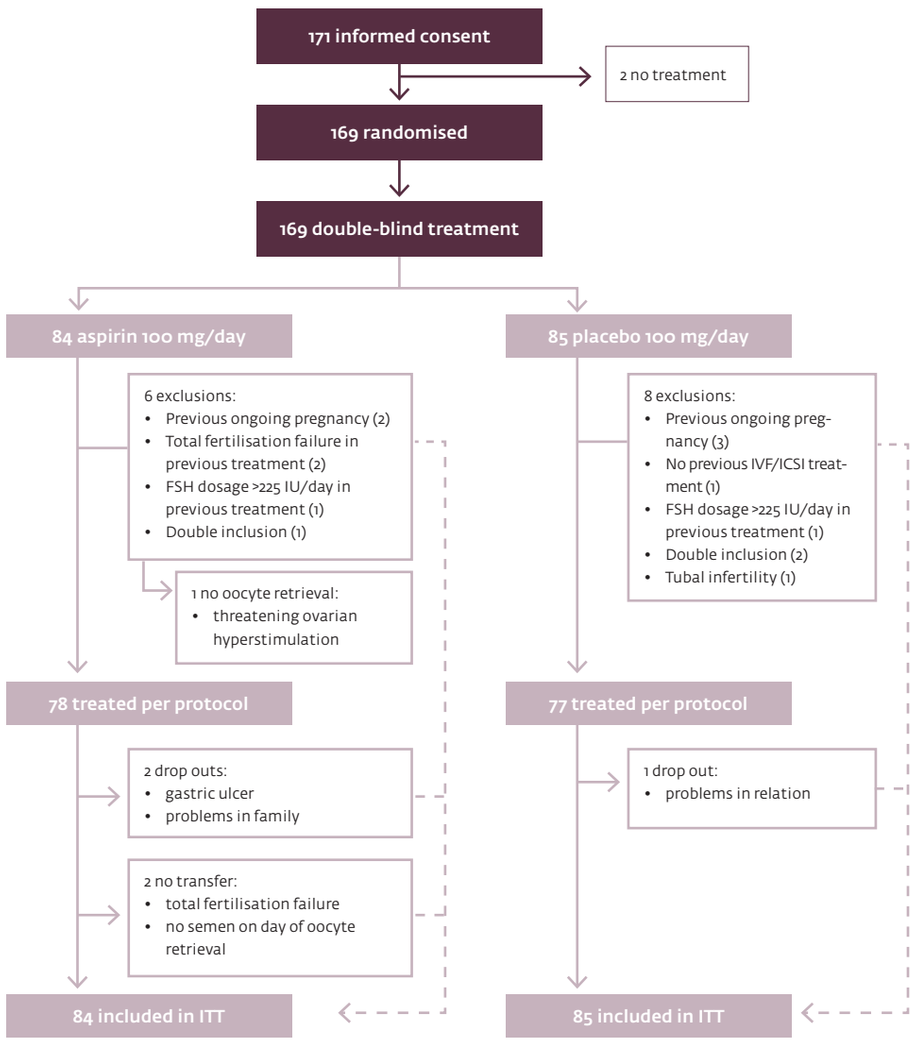
For this prospective randomised double-blind placebo-controlled trial, we included patients who met the following criteria: < 39 years of age at the start of treatment, with serum FSH level ≤ 10 IU/l on cycle day 3 and with at least one previous IVF/ICSI treatment with failed conception. In this previous treatment cycle, the patients had been given a maximum daily dosage of 225 IU FSH resulting in at least 4 oocytes at oocyte retrieval. Patients did not have a previous ongoing pregnancy, both ovaries were present and there was no contra indication for aspirin.
Patients were excluded if they had tubal pathology, ovarian hyperstimulation syndrome in a previous treatment cycle, body mass index > 30, smoking more than 5 cigarettes per day, untreated endocrinopathy, systemic disease, hypertension, previous allergic reaction to study medication or contra indication for pregnancy.
Patients were all treated with a long GnRH-agonist protocol with oral contraceptives as pretreatment, as previously described [15]. Ovarian response and endometrial thickness were monitored by vaginal ultrasonography and serum estradiol determinations. Human chorionic gonadotrophin 10.000 IU s.c. was administered when there was at least 1 follicle ≥ 18 mm and in total 3 of more follicles ≥ 16mm.
Ultrasonographic directed oocyte retrieval was performed 36 hours later. Embryo transfer was generally performed on day 3 after oocyte retrieval. If there was no possibility for embryo selection, embryo transfer was performed on day 2 after oocyte retrieval. In consultation between physician and the couple, 1 or 2 embryos were transfered.
A serum pregnancy test was performed 14-16 days after oocyte retrieval. Pregnancies were monitored by transvaginal ultrasonography at 5, 6, 9 and 12 weeks gestational age.
Randomisation was performed by an independent pharmacist of the hospital pharmacy using computerised tables. Randomisation numbers corresponded with the numbers on the medication boxes containing 80 identical-looking tablets of either 100 mg aspirin or 100 mg placebo. These medication boxes were filled by an independent person at the hospital pharmacy, assuring that both patient and investigator were blinded to the randomisation and the content of the tablets.
The numbered boxes were handed out consecutively after patients had given their written informed consent at the start of the treatment cycle. All patients started the study medication together with the start of the oral contraceptive pill and continued this daily medication until the day of pregnancy test.
Pregnant patients continued the study medication until 12 weeks' gestational age. For these patients a follow up medication box containing 50 additional tablets was distributed by the hospital pharmacy. This follow-up medication contained the same medication to which the patient had initially been randomised. Concealment was maintained in the follow-up medication.
The pulsatility index (PI) is commonly used as an index of blood flow velocity [16]. In this study, the blood flow velocity in the uterine arteries was monitored throughout the treatment by measuring the pulsatility index (PI) and the resistance index (RI) of both arteries at all visits: at the start of the treatment, at each ultrasonographic monitoring of follicle growth, on the day of oocyte retrieval, on the day of embryo transfer, one week after oocyte retrieval and on the day of the pregnancy test. In case of pregnancy we continued measurements at 5, 6, 9 and 12 weeks' gestational age. All measurements were performed by one physician.
After the study had been finished, patients received a questionnaire that inquired the following: Did they have any idea during the treatment whether they were taking aspirin or placebo (if yes: why)? Did they take the study medication daily (if no: why)? Did they use additional aspirin (if yes: how often and in what dosage)? Did they have additional therapy (eg acupuncture)? Did they consider taking aspirin in a following treatment? Did they take aspirin in a following treatment and why? Would they use aspirin in a following treatment, and why? Did they tell others about the study (if yes: positively, negatively or purely informative).

Sample size of 75 patients per treatment group was calculated based on a 20% increase in clinical pregnancy rate, defined as percentage of intact intra uterine pregnancies at 6 weeks' gestational age, in favour of the group treated with aspirin with α= 0.05 and β=0.10 [6]. Statistical analyses between the groups were performed using t-tests and χ^2 tests. Multivariate regression analysis was performed to analyse whether administration of aspirin was associated with clinical pregnancy. Both intention-to-treat and per-protocol analyses were performed.
To investigate the relation between treatment and pulsatility index (PI) through time multilevel analyses were applied. The technique of multilevel analysis takes into account that the same subjects are repeatedly measured and uses all available data, irrespective of the number of repeated measurements. This indicates that missing observations are allowed. Furthermore, multilevel analysis is capable of dealing with irregular time intervals [17]. For these analyses we have used a random intercept model with 2 levels (patient as level 2 and time as level 1). As independent variables, we used the dummy variable indicating treatment, time and the interaction between time and treatment to see whether the effect of time differed for each treatment. We also considered non-linear

effects of time by including the second and third power of time in the model. For each dependent variable, we choose the best fitting model based on the two-log likelihood.
Analyses were done using SPSS 14.0 (SPSS Inc, Chicago, Il) and MLWin 2.0 software (MLWIN University, Bristol, UK).

The study was approved by the institutional review board of the VU University medical center; all patients gave their written informed consent.
The trial is registered with the Dutch Trial Register and as an International Standard Randomised Clinical Trial, number ISRNCTM97507474.

Figure I.
Flowchart



Results

A total of 169 patients were included in this double-blind randomised controlled trial. Fourteen patients had to be excluded (Figure I). These patients are part of the intention to treat (ITT) analyses, but were left out in the per protocol (PP) analyses. Baseline characteristics were not significantly different between the treatment groups (Table I).
There were no differences between the treatment groups regarding treatment characteristics: duration of stimulation, daily FSH dosage, number of follicles on the day of Pregnyl (hCG)-injection, endometrial thickness, serum level of estradiol, number of oocytes retrieved, fertilisation rate, number of embryos available for transfer, number of embryos transfered, the Cumulative Embryo Score (CES) of the morphological most optimal embryo, percentage of patients with possibility of cryopreservation and number of embryos cryopreserved (Table I).

Thirty-two patients in the aspirin group (40.5%) and 33 patients in the placebo group (39.3%) had serum hCG >50 IU/L, p=0.874. At 6 weeks' gestation, 33 patients in the aspirin group (41.8%) and 31 patients in the placebo group (36.9%) had an intra uterine pregnancy, p=0.525. There were respectively 5 (15.2%) and 9 (28.1%) pregnancies with double implantation, p=0.203. The implantation rate was 26.6% in the aspirin group and 26.8% in the placebo group, p= 0.971. Five patients in the aspirin group (15.2%) and 6 patients in the placebo group (18.8%) had total pregnancy loss, p=0.699. There was one vanishing twin in the aspirin group and one patient in the placebo group had an ectopic pregnancy. At 12 weeks' gestation, 28 patients in the aspirin group (35.4%) and 26 patients in the placebo group (31.0%) had an ongoing pregnancy, p=0.677. In the aspirin group 4 patients (12.1%) and in the placebo group 9 patients (28.1%) had an ongoing twin pregnancy, p= 0.202 (Table II). When a subgroup analysis was performed for patients with unexplained subfertility we also found no significant differences in pregnancy rates. (Data not shown).

Table I
Baseline characteristics and characteristics of treatment cycles (Intention to treat analysis).

	Aspirin (N=84)	Placebo (N=85)	P
Age (years)	33.04	32.96	NS
FSH on cycle day 3 (IU/L)	6.16	5.96	NS
BMI	22.94	23.00	NS
Dosage in previous treatment (IU/day)	184.3	184.0	NS
N° of oocytes in previous treatment	12.9	13.6	NS
N° of previous treatments*	1.8	1.7	NS
ICSI-treatment (%)	60.7	65.9	NS
Primary infertility (%)	76.2	76.5	NS
Indication for treatment			NS
Male factor infertility (%)	66.7	64.7	
Idiopathic infertility (%)	23.8	28.2	
Other indications (%) **	9.5	7.1	
Stimulation dosage (IU/day)	199.3	195.8	NS
Duration of stimulation (days)	11.5	11.4	NS
N° of follicles	16.0	17.0	NS
Endometrial thickness on day of hCG-injection (mm)	10.0	10.6	NS
Serum estradiol on day hCG-injection (IU/L)	8070	7725	NS
N° of oocytes retrieved	13.7	13.5	NS
Fertilisation rate (%)	62.8	63.4	NS
N° of embryos available for transfer	7.48	7.48	NS
Average No of cells per transfered embryo	7.1	6.7	NS
CES of the morphological most optimal embryo	9.06	8.96	NS
N° of embryos transfered	1.89	1.86	NS
Cryopreservation possible (%)	28.6	25.9	NS
N° of embryos cryopreserved***	4.6	5.6	NS

* including cryopreservation treatment
** other indications: endometriosis, fertilisation failure in IVF, hormonal cause, cervical hostility and one patient with tubal factor (excluded from per protocol analyses).
*** Average for the group of patients with possibility of cryopreservation.
CES= cumulative embryo score,
NS= not significant

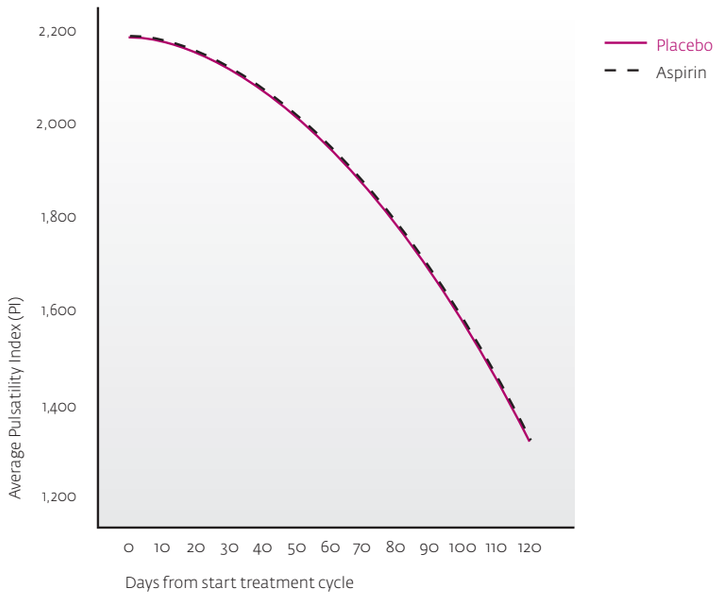
Table II
Pregnancy rates (intention to treat analysis).

	Aspirin (N=84)	Placebo(N=85)	P
Serum hCG> 50 IU/L (%)	40.5	39.3	NS
Intra uterine pregnancy at 6 weeks gestation (%)	41.8	36.9	NS
Implantation rate (%)	26.6	26.8	NS
Total pregnancy loss (%)	15.2	18.8	NS
Ongoing pregnancy at 12 weeks gestation (%)	35.4	31.0	NS
Multiple pregnancy (%)	12.1	28.1	NS

NS= not significant

The relation between treatment and Pulsatility Index (PI) was analyzed by means of multilevel analyses, using a random intercept model with two levels. From these analyses we found that there was no effect of treatment on the PI. We also found that through time PI decreases in a non-linear manner. The effect of time was not different for the two treatment groups. The amount of decrease through time was also not different between the groups. The course through time of the PI_{right} and PI_{left} could best be described as parallel, non-linear and decreasing (Figure II).

Figure II.
Pulsatility Index (PI) through time. The black dotted line represents patients treated with 100 mg of aspirin daily; the red continuous line represents patients treated with 100 mg of placebo daily.



After 2 months, 120 questionnaires (71%) had been filled out and returned, another 8 (4.7%) had been returned to sender. The remaining 41 patients received the questionnaire for a second time. After that we received another 14 questionnaires. The total response rate was 79.3%. Significantly more patients who were treated with aspirin suspected that they had been treated with aspirin (Table III). As a reason for their suspicion, 8 patients in the aspirin group and 2 patients in the placebo group reported that they had more bruises or bled easier after injecting the hormonal medication during this treatment. Other reported reasons were: gut feeling, this treatment was different, this treatment resulted in pregnancy, no side-effects, and no complaints of headaches during this treatment.

Table III
Questionnaire: Idea of the treatment

	Aspirin	Placebo	P
Did you have an idea whether you took aspirin or placebo tablets during the treatment?			0.014
No idea at all	69%	83.9%	
Thinks aspirin treatment	29.3%	9.7%	
Thinks placebo treatment	1.7%	6.5%	

Fifty-nine patients had a following treatment, 22 of them considered using aspirin in that treatment, 11 patients did use aspirin in the following treatment and 3 of them became pregnant.
Sixty-seven patients (55.8%) would take aspirin in a following treatment if it was proven that it is beneficial for their chance of pregnancy, 29 patients (24.2%) would take aspirin in a following treatment regardless of scientific proof. Forty-two patients had told others that they were participating in this study, and 6 of these patients had done so as an advice.

Discussion

Our study is the first sufficiently powered study demonstrating that administration of low-dose aspirin in IVF/ICSI treatment does not improve clinical pregnancy rates for patients with previous failed conception. Besides the clinical pregnancy rate, there were no other outcome measures that improved in the group of patients treated with low-dose aspirin. Most importantly, we found that the pulsatility index of the uterine arteries was not influenced by the treatment patients received, indicating that aspirin does not effect the uterine blood flow.
Embryo implantation, and therefore the chance of pregnancy after IVF/ICSI treatment, is a result of several factors[18], most importantly of the receptiveness of the endometrium and the quality of the embryo(s)[15]. As an inhibitor of tromboxane-A_2, aspirin inhibits vasoconstriction and would therefore improve the uterine blood flow[6]. This improvement of the uterine blood flow would help to optimize the endometrium. In our study, we found no evidence for this hypothesis. We found no differences in the change of the pulsatility index (PI) of the uterine arteries, which we used as a measure of the uterine blood flow. The decline in PI, and therefore the increase in blood flow, was the same for both treatment groups. Using multilevel analyses, we demonstrated that the PI was not affected by the treatment patients received. Therefore, we conclude that changes in PI throughout the IVF treatment are the result of natural changes within a menstrual cycle combined with the effect of the IVF treatment itself[16].
It has also been suggested that the development of the endometrium may improve in aspirin-treated patients groups[6]. In our study, we did not find statistical differences regarding the endometrial thickness. Therefore, we conclude that any improvement of the endometrium is not reflected by the endometrial thickness. Ideally, the endometrial quality should be analysed by means of an endometrial biopsy. Because the optimal timing of such a biopsy would be in the implantation window, with the risk of interfering in the implantation process, it would be very difficult to find ethical approval for studies with such interventions.
Regarding the embryos, that seem to be the most important players in the process of implantation, we also found no evidence for a positive effect of aspirin treatment. In this study, we found no differences in numbers of embryos available for transfer, possibility of cryopreservation and number of embryos that could be cryopreserved. We also looked at cumulative embryo score (CES)[15] of the morphological most optimal embryo and found that the embryo quality of the morphological most optimal embryo was not different between the groups. Quantitively and morphologically, the quality of the embryos is not influenced by the administration of aspirin.

With these results, we join the discussion on the use of aspirin in IVF. Since the positive reports on aspirin use in patients with tubal infertility[6], several studies have analysed the potential benefit for the IVF patient population[9-14]. There is large heterogeneity among these studies[19], possibly because most of the studies had selected different patient groups and used different periods of aspirin administration. Moreover, a number of these studies did not present sample size calculations and when there was a sample size calculation this number was not always reached.

In the past year, there were three meta-analyses[20-22] published that tried to sum up the results of these studies. As a result of different selection criteria and different models of analysis, these meta-analyses still do not supply a final answer. The results of our study would lessen the overall effect reported by each of the meta-analyses, regardless of their selection criteria or model of analysis, but it would not alter significance as such. Besides this, the question remains at which period in the treatment aspirin should be used and whether the effect of low-dose aspirin administration in IVF may only truly count for certain subgroups of patients.

Recently a Cochrane review[19] found no significant improvement in pregnancy rates when IVF was combined with low-dose aspirin administration. The authors suggested that women with a history of implantation failure or unexplained subfertility would be subgroups worthy of further investigation. With the present study, we specifically addressed the first group and again found that administration of low-dose aspirin is not beneficial with regard to clinical pregnancy rates and changes in pulsatility index.

We expect that the discussion on the use of aspirin in IVF will continue. In the present study, we demonstrate that aspirin administration is not beneficial for non-tubal IVF patients with previous failed conception. Moreover, we demonstrate that the assumption that aspirin would improve the uterine blood flow is false.

Besides this we learned from the answers to the questionnaire in our study that the trust and belief in the potential of aspirin is very high among patients. More than one-half of the patients in our group would take aspirin in a following treatment, if there is proof that it is beneficial for their chance of pregnancy. One-quarter of the patients reported that they would take aspirin in a following treatment regardless of scientific proof, and a couple of these patients even had already done so. One of the explanations for this high trust in aspirin may be the fact that aspirin is one of the best known drugs throughout the world and throughout history. In addition, this is a group of patients with a long-unanswered desire, who are willing to take a small accountable risk. Because aspirin is such a well known and easy accessible drug, we think these replies should raise some concern and carefulness among doctors working in the IVF

field. It illustrates the importance of patient education, especially when even evidence based medicine does not supply us with solid answers.

Acknowledgements

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9

Lower incidence of hypertensive complications during pregnancy in patients treated with low-dose aspirin during in-vitro-fertilisation and early pregnancy.

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Abstract

Background: The use of aspirin during in-vitro fertiflisation (IVF) has been investigated for its effect on pregnancy rates after IVF. In most of these studies, aspirin administration was then prolonged throughout the first trimester of pregnancy. By inhibiting vasoconstriction, use of low-dose aspirin in the first trimester could influence placentation and therefore prevent or delay development of hypertensive pregnancy complications, such as pregnancy induced hypertension (PIH) and pre-eclampsia (PE).

Methods: This study involved the follow-up by questionnaires and hospital records of patients with an ongoing pregnancy in a prospective randomised double-blind placebo-controlled trial on the effect of low-dose aspirin during IVF. Aspirin treatment was continued throughout the first trimester of pregnancy. The primary endpoint of this follow-up study was the incidence of pregnancy complications. The original trial is registered with the Dutch Trial Register and as an International Standard Randomised Clinical Trial, number ISRNCTM97507474.

Results: There were 54 patients who had ongoing pregnancies in the original trial; 90.7% returned the questionnaire and all Dutch hospital records were retrieved. A significant difference in pregnancy complications was found in the incidence of hypertensive pregnancy complications: 3.6% in the aspirin group and 26.9% in the placebo group ($p<0.05$), resulting in number-needed-to-treat (NNT) of 10.3 to prevent hypertensive complications in one pregnancy after IVF treatment.

Conclusion: The incidence of hypertensive complications was significantly lower in the group of women treated with low-dose aspirin throughout IVF treatment and first trimester of pregnancy. These results suggest a potential benefit of low-dose aspirin during IVF and first trimester to prevent hypertensive pregnancy complications. The findings justify further investigation in placebo-controlled randomised trials.

Introduction

Pregnancy induced hypertension (PIH) and pre-eclampsia (PE) are common complications in pregnancy. About 10 to 15% of pregnancies are complicated by these hypertensive disorders, making them one of the most important causes of maternal and neonatal morbidity and mortality[1]. Some studies concluded that in in-vitro-fertilisation (IVF) patients the risk of developing pregnancy induced hypertension is twofold[2] and that the risk of pre-eclampsia is even 2.7 times increased[3]. Therefore women undergoing IVF or intra-cytoplasmatic-sperm-injection (ICSI) treatment may be more prone to develop hypertensive complications[3-5], not only because of their higher maternal age and nulliparity, but probably also because of abnormal placentation[4].

The basis of hypertensive complications in pregnancy is founded in the early stages of pregnancy[6]. The process of placentation is essential: poor placentation leads to the development of PIH and PE later in pregnancy[6;7]. The cytotrophoblast invades the maternal spiral arterioles as early as 8 weeks of gestation. In PE, this trophoblast invasion of the uterine spiral arterioles is shallow, leading to decreased placental perfusion and therefore placental ischaemia. In later stages of pregnancy the placenta secretes antiangiogenic factors, causing maternal systemic endothelial dysfunction responsible for the clinical aspects of PE: hypertension, proteinuria, coagulopathy and liver dysfunction[7]. As an inhibitor of the cyclo-oxygenase (COX) enzyme aspirin inhibits thromboxane A_2 and reduces vasoconstriction. Therefore, the use of low-dose aspirin has been evaluated as a potential therapy for prevention or delay of the development of pre-eclampsia. Systematic reviews show moderate but consistent reduction of PE, but numbers-needed-to-treat were very high[8-10].

The use of aspirin during IVF has been investigated for its effect on pregnancy rates after IVF[11-13]. In most studies, aspirin administration was prolonged throughout the first trimester of pregnancy, which could influence placentation and prevent or delay development of hypertensive pregnancy complications, such as pregnancy induced hypertension (PIH) and pre-eclampsia (PE).

Recently, we performed a randomized placebo-controlled double-bind trial evaluating the effect of low-dose aspirin during IVF or ICSI treatment on the pregnancy rate[13]. We found no significant improvement of pregnancy rate for patients using low-dose aspirin throughout the IVF treatment. The aim of the current study was to compare the incidence of hypertensive pregnancy complications among the pregnant patients from this previous trial.

Materials & Methods

Follow-up was made of those patients who had an ongoing pregnancy in the prospective randomised double-blind, placebo-controlled trial investigating the effect of low-dose aspirin on pregnancy rates among IVF/ICSI patients with previous implantation failure that was described previously [13]. In the trial, patients were allocated to Group A (Aspirin) or Group B (Placebo) and started the study medication (100 mg aspirin or 100 mg placebo) simultaneously with the start of the oral contraceptive pill and continued this daily medication until the day of the pregnancy test. Pregnant patients continued the study medication until 12 weeks of gestational age.

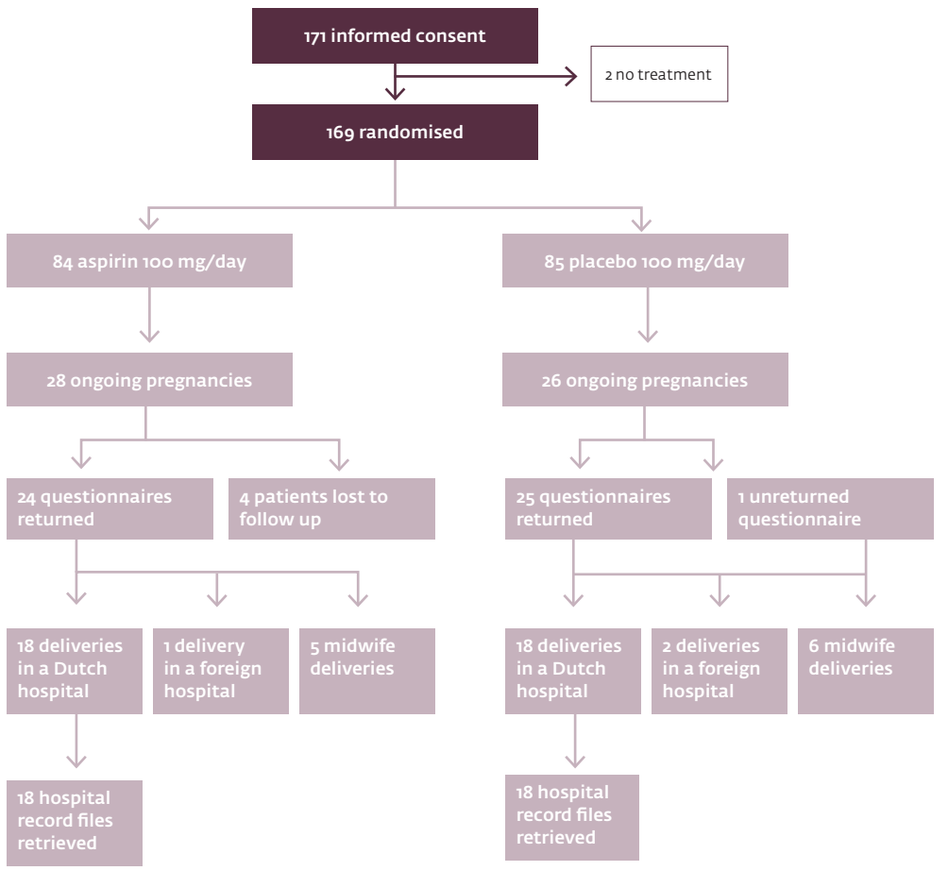
After delivery, all patients received a questionnaire inquiring about the location of the delivery, complications during the pregnancy and delivery and about the health of their babies. Hospital record files were collected of all patients who delivered in a Dutch hospital under guidance of a doctor. In the Netherlands, patients with an uncomplicated pregnancy can have a delivery at home or in a hospital under the guidance of a midwife. We checked with the local hospitals about whether these patients really did not attend medical care. Data from the questionnaires were linked with data from the hospital records, which were also checked for complications during the pregnancy and delivery and for the health of the babies.

Pregnancy induced hypertension (PIH) was defined according to national guidelines as systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg on repeated measurements after 20 weeks gestation. Pre-eclampsia (PE) was defined as pregnancy induced hypertension and proteinuria (≥ 300 mg/24 hours). In case of haemolysis, elevated liver enzymes and low platelets patients were diagnosed with HELLP-syndrome. Small for gestational age (SGA) was defined as birth weight below the 5th percentile. First trimester blood loss was defines as vaginal blood loss before 12 weeks gestational age not related to miscarriage. Post partum haemorrhage was defined as ≥1000 ml blood loss in 24 hours.

Data were analysed using Student's t-test, Fisher's exact test and chi-squared test where appropriate (Statistical Package for Social Science (SPSS) 15.0, SPSS Inc, Illinois, USA).

The study was approved by the institutional review board of the VU University medical center and all patients gave their written informed consent. The original trial is registered with the Dutch Trial Register and as an International Standard Randomised Clinical Trial, number ISRNCTM97507474.

Figure I.
Flowchart of the aspirin trial and follow-up study.



Results

The original trial included 169 patients, of whom 54 had an ongoing pregnancy (31.9%). Of these pregnant patients, 28 were treated with aspirin (group A) and 26 received placebos (group B); the pregnancies included four and nine sets of twins, respectively. Baseline and pregnancy characteristics are reported in Table I. All 54 patients received a questionnaire after delivery and 49 patients (90.7%) completed and returned the questionnaire. We retrieved full records of one non-responder while four were lost to follow up. A flow chart of patients is shown in Figure I. There were 11 patients who remained fully under care of their midwife; their pregnancies and deliveries were uncomplicated, as was confirmed by the local hospitals. Six of them delivered at home and five patients delivered in a hospital under guidance of a midwife. There were 39 patients who delivered their babies under guidance of a doctor, either because of a medical history or because of complications during pregnancy or during delivery that required medical attention. Three patients delivered in a foreign hospital: two in the United Kingdom and one in Thailand. The patients who delivered under guidance of the midwife had no hospital patient record file. We collected all Dutch hospital patient record files (Figure I).

Table I
Baseline and pregnancy characteristics

	Aspirin (N=28)	Placebo (N=26)	P
Age (years)	32.8	32.6	0.81
FSH on cycle day 3 (IU/L)	5.85	5.88	0.93
BMI	22.75	22.72	0.97
Primary infertility (%)	78.6	73.1	0.64
Twin pregnancy	4 (14.3%)	9 (34.6%)	0.08
Caesarean section (%)	52.0	42.3	0.49
Gestational age at delivery (weeks)	38.3	37.9	0.61
Birthweight (kg)	3.156	3.089	0.76

Table II
Complications during pregnancy

	Aspirin (N=28)	Placebo (N=26)	P
First trimester blood loss	2 (7.1%)	3 (11.5%)	0.38
Post partum haemorrhage	1 (3.6%)	0	0.49
Hypertensive complication	1 (3.6%)	7 (26.9%)*	0.02
Pregnancy Induced Hypertension	1	4	
Pregnancy Induced Hypertension with pre-eclampsia	0	1	
Pregnancy Induced Hypertension with HELLP	0	2	
Premature contractions/ premature rupture of membranes	5 (17.8%)	4 (15.4%)**	0.62
Premature delivery***	4	2	
Growth restriction/growth difference (twins)	1 (3.6%)	1(3.8%)	1.00
No complications	14 (50%)	11 (42.3%)	0.26
Lost to follow up	4 (14.3%)	0	0.11

* Including 3 twin pregnancies.
** One patient was admitted to the hospital twice, she also had a pulmonary embolism during her pregnancy.
*** Including 2 twin pregnancies in each group.

Table II reports all complications. In the aspirin group, 14 patients, including one twin pregnancy, had uncomplicated pregnancies. Two patients reported first trimester blood loss; one twin pregnancy had postpartum haemorrhage. One patient had pregnancy induced hypertension (PIH) without need for antihypertensive treatment. Three pregnancies, including one twin pregnancy, were complicated by blood loss combined with premature contractions at 29, 32 and 34 weeks gestational age respectively. One other twin pregnancy was delivered prematurely at 34 weeks gestational age after premature rupture of membranes. One patient had premature contractions but was successfully suppressed and she delivered her baby at 38 weeks gestational age. One child was small for gestational age.

In the placebo group, 11 women, including one patient pregnant with twins, had no complications during pregnancy. Three patients reported first trimester blood loss, though only one such case was confirmed by her medical record. Seven (26.9%) patients reported PIH, PE or HELLP-syndrome, including 3 patients pregnant with twins. Two patients had PIH only, one patient pregnant with twins had PIH combined with cholestasis, and one patient pregnant with twins was treated with magnesium sulphate, but had no pre-eclampsia. Three patients had PIH with pre-eclampsia, and two of them, including one twin, developed HELLP-syndrome. One twin pregnancy was complicated by a pulmonary embolism and premature contractions. One twin pregnancy was complicated

by a growth difference between the babies, resulting in a delivery at 36 weeks of a boy (2250 gram) and a girl (3320 gram). One pregnancy was complicated by blood loss and premature contractions, and two twin pregnancies had premature contractions and premature delivery at 31 and 34 weeks. One patient pregnant with twins reported premature contractions, but she was never admitted.
There was a statistically significant difference between the groups with regard to the incidence of hypertensive complications during pregnancy, with a higher incidence in the placebo group, 3.6% vs 26.9%, $p<0.05$ (Table II).
Twelve patients in the aspirin group and fifteen patients in the placebo group had a vaginal delivery. Caesarean section was performed for the following reasons: non cephalic position of the baby (n=10), fetal distress (n=4), slow progression of delivery (n=2), big baby (n=2), high position of the babies head (n=2), pregnancy cholestasis (n=1) and meconium in the amniotic fluid (n=1). Fifteen children (4 twins and 7 singletons) were admitted to the neonatal care unit, because of premature delivery, maternal fever, jaundice or low glucose levels. None of the children died. No permanent handicaps or diseases were reported. One child was later diagnosed with neurofibromatosis.

Discussion

At least one out of ten pregnancies is complicated by hypertensive complications. Pregnancy induced hypertension can develop into pre-eclampsia, eclampsia or even HELLP-syndrome; these are an important cause of maternal and neonatal morbidity and mortality[1]. In this pilot study, we compared the incidence of hypertensive complications in pregnancy among the pregnant patients of a prospective randomised trial, in which they used aspirin or placebo daily during IVF treatment and first trimester of pregnancy. We found a much lower incidence of hypertensive complications in women treated with low-dose aspirin.
Acetylsalicylic acid, or aspirin, acelytates cyclo-oxygenase through which it inhibits the synthesis of several prostaglandins, prostacyclins and thromboxane-A_2. When administered in low dosage the effect of aspirin more selectively inhibits tromboxane-A_2, which induces platelet aggregation and vasoconstriction. Since pre-eclampsia is a condition with excessive production of tromboxane-A_2[8;14] the use of low-dose aspirin in pregnancy has been investigated for its potential ability to prevent or delay the development of pre-eclampsia.
Our finding of lower incidence of hypertensive complications in pregnancy for women treated with low-dose aspirin is in line with previous findings of reduced incidence of pre-eclampsia[8;10] and reduction of blood pressure[15].
Aspirin treatment in pregnancy for the prevention of pre-eclampsia is a known approach, but the novelty of the present study is the very early stage at which aspirin treatment was started, before any knowledge of conception, therefore during implantation and placentation. In most other studies patients were treated with aspirin from 20 weeks gestational age. It has been suggested that the crucial time for starting treatment may be before 16 or even before 12 weeks gestational age[8].
Apparently, early aspirin treatment at the time of placentation influences invasion of the maternal spiral arterioles in a crucial way. In pre-eclampsia, trophoblast cells produce more thromboxane-A_2 leading to vasoconstriction[16].
One mechanism could be that early aspirin treatment prevents the first stage of abnormal placentation[7], which is marked by increased tromboxane-A_2 production by the trophoblast cells[17]. The inhibiting effect of aspirin on production of tromboxane-A_2 could result in less vasoconstriction and, as a consequence in this stage of placentation, in more optimal invasion of the uterine spiral arterioles. Subsequently, improved placental perfusion may reduce the chance of developing PIH or PE.
Meta-analyses on aspirin for the prevention of pre-eclampsia conclude that aspirin treatment results in a moderate but consistent reduction of PE, but the numbers-needed-to-treat (NNT) are high; point estimates are NNT=100[8], although they may vary for different subgroups[10], since some groups of women

may have a higher a priori risk. Some studies have found women pregnant after IVF treatment to have a twofold risk of developing pregnancy induced hypertension[2] and 2.7 times increased risk of developing pre-eclampsia[3], making them an interesting group for the potential preventive effect of aspirin on pregnancy complications.
The potential benefit of aspirin administration during IVF treatment has been investigated in the past decade with various results. The overall estimate of the number-needed-to-treat is around 25[18]. None of the previous studies have addressed pregnancy outcome in terms of hypertensive complications. With the current findings, we calculated a NNT of 10.3 to prevent one pregnancy with hypertensive complications after IVF treatment. This suggests that the beneficial aspect of treating IVF patients with low-dose aspirin is not necessarily to increase their chance of pregnancy, but to decrease their chance of hypertensive complications during pregnancy once achieved.
Regarding other pregnancy complications we found no significant differences between the aspirin and placebo group. Incidences of premature contractions, premature delivery and growth restrictions were comparable between the groups. Interestingly, we did not find that differences regarding the incidence of first trimester blood loss, especially since aspirin inhibits platelet activity and therefore prolongs bleeding time.
By combining the questionnaires with data from the hospital records we tried to rule out bias as a result of incomplete report on complications, still it is possible that not all complications were diagnosed and reported. We stress that our study is only a small pilot study, which was not powered for this particular outcome. One explanation for the significant difference in the incidence of hypertensive pregnancy complications between the aspirin and placebo groups would be a possible higher prevalence of twin pregnancies in the latter. Nevertheless, the current findings justify further investigation in power-calculated placebo-controlled randomised trials. Based on the incidence of hypertensive pregnancy complications in this trial, to detect a difference of 20% with α= 0.05 and β=0.80, seventy pregnant patients per treatment group would need to be included in a randomized placebo controlled trial. Bearing in mind that the incidence of hypertensive pregnancy complications in the placebo group was rather high, the true difference between the groups may be smaller: if the difference is only ten percent each group needs to consist of 190 pregnant patients.
In summary, in this pilot study we reported the incidences of pregnancy complications of women who had an ongoing pregnancy after participation in a double blind placebo controlled randomized trial, in which low-dose aspirin or placebo was administered daily throughout the IVF treatment and first trimester of pregnancy. We found a significantly lower incidence of hypertensive pregnancy complications in the aspirin treated group; therefore we suggest further investigation in randomized placebo controlled trials.

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10 General discussion

General discussion

Embryo implantation has remained a 'black box' in pregnancy[1], regardless of whether it is a pregnancy after spontaneous conception or a pregnancy achieved through assisted reproductive techniques. Unravelling the secrets of the implantation process is now one of the main focuses for researchers within the field of reproductive medicine. Gradually, we come to understand more of the implantation factors and of the circumstances under which the process takes place[2]. Extending our insight in the process of embryo implantation can potentially provide us with possibilities to influence implantation factors or the circumstances of the implantation process and this it will hopefully help to improve pregnancy rates both after spontaneous conception and after artificial reproductive techniques.
The central aim of this thesis was to gain more understanding in the complex process of embryo implantation. Since in artificial reproductive techniques (ART), such as in-vitro-fertilisation (IVF) and intra-cytoplasmatic-sperm-injection (ICSI), the timing of the implantation process is precisely known and ART offers an ideal model for the investigation of embryo implantation, we focused our studies on in-vitro-fertilisation treatment. We investigated predetermined patient related variables as well as treatment related -and therefore adjustable- variables (such as techniques and medication) for their contribution in the implantation process.

Human chorionic gonadotrophin

We started this thesis by demonstrating that it is possible to predict the viability of a pregnancy rather accurately based on a single serum measurement of human chorionic gonadotrophin (often referred to as pregnancy hormone) only 14 or 15 days after oocyte retrieval[3], corresponding with the day the next period should have started in a spontaneously conceived pregnancy. Even though several studies have looked for other and potentially more precise markers for viability of pregnancies[4-7], hCG has remained a reliable, easily accessible and affordable marker for viability. The level of this pregnancy hormone in maternal serum can be regarded as a reflection of strength of the implantation of the embryo(s) and therefore as a result of all variables that determined the success of the embryo-endometrial dialogue.

Multiple implantation

Multiple implantation represents a situation in which the implantation process takes place in duplicate. As the chance of embryonic loss is lower in first trimester multiple pregnancies[8], the quality of multiple implantation may be very different from single implantation and therefore the circumstances and conditions under which multiple implantation takes place may be very different

as well. In a group of IVF and ICSI patients who had a pregnancy after double embryo transfer (DET), it was possible to investigate multiple implantation itself. We analysed predetermined factors for their contribution to multiple implantation and for their contribution to continuation of the pregnancy. We demonstrated that when multiple embryos enter the uterus for implantation their chance of success at 6 weeks of gestation is predominantly determined by embryonic factors, whereas continuation of a pregnancy through the rest of the first trimester is a result of the combination of embryonic potential and uterine environment[9].
As for many other biological qualities, receptivity of the endometrium for implantation may be a quality with a hereditary basis, the chance of success of implantation may therefore be predetermined just by a person's hereditary background. Knowing that multiple pregnancy has a hereditary basis[10], we wanted to investigate to what extent multiple implantation has a hereditary basis, as this may be a reflection of high receptivity of the endometrium. In the same group of patients with DET after IVF or ICSI[9] we analysed the role of a hereditary basis as a contributor to multiple implantation and to pregnancy continuation. For this purpose we used a questionnaire that inquired after a family history of twinning[11]. We found that a positive family history of twinning does not contribute to the chances of multiple implantation or continuation of the pregnancy throughout the first trimester. We therefore conclude that the hereditary basis for multiple pregnancy is restricted to the chance of multiple ovulation only[12].
The success of implantation is predominantly determined by embryonic factors; the uterine environment does not influence the chance of multiple implantation and only influences pregnancy continuation combined with embryonic potential. Bearing these conclusions of our first studies in mind we can only conclude that the (transfered) embryo has a key role in determination of success in implantation. Further research within the field of embryo selection will help us determine more adequately which embryo has the best intrinsic potential. Currently we select embryos by analysing them for their morphology[13], but embryos that are morphologically equal do have different potential for establishing pregnancy. Recent studies have focused on genetic analysis or metabolomic profiling of embryos. Especially since genetic analysis of embryonic cells was proven do be disastrous for pregnancy results[14], metabolomic profiling of embryos before selection for transfer[15] is a potentially promising technique, which will hopefully bring us closer to understanding the process of embryo implantation.

External influences

The success of embryo implantation seems largely determined by qualities of

the embryo and of the endometrium. In artificial reproduction techniques a third player, the IVF-clinician, is involved, who has possibilities to intervene and possibly influence the implantation process; for instance by changing the technique of the embryo transfer[16;17] or altering medication used during treatment[18].

The moment of embryo transfer is literally the final step within reach of the clinician and therefore a final option to physically influence the circumstances of implantation. Not only the type of catheter can change the chance of success[17], but also the depth of catheter placement can influence success[19]. Controlling the depth of catheter placement would be more precise when the transfer is performed under ultrasonographical guidance. We compared the pregnancy rates of transfers performed under ultrasonographical guidance and transfers based on uterine cavity depth and learned that pregnancy rates were not different between these groups. Therefore, ultrasonographical guidance is not essential for control on the depth of catheter placement at transfer[20].

Ultrasonographic guidance at embryo transfer does offer the possibility to visualise the transfer air bubbles[20], opening new perspectives of research. This way it was possible to illustrate that the content of the transfer catheter is not influenced by immediate ambulation after embryo transfer, supporting previous studies demonstrating that pregnancy rates are not influenced by immediate ambulation after embryo transfer[21;22]. Although the air bubbles are only regarded as an indication for the embryos, our study illustrates that the uterine environment is a secluded environment and that the implantation process is only subject to the interplay between embryo and endometrium.

Analysing the results of the study comparing ultrasonographic guided transfer with uterine length measurement guided transfer[20]; we also found that pregnancy rates were higher when the transfer content ended in the upper part of the thick part of the endometrium (endometrial plate)[23]. This may be a reflection of differences in endometrial receptiveness or difference in expression of certain implantation factors essential for an optimal embryo-endometrium dialogue and may therefore lead us to ways of influencing the chances of pregnancy. At this moment, we do not (yet) have a possibility to direct the transfer content. When we have an opportunity to predetermine post transfer embryo position in the uterus rather than just 'launching them off', we will have the possibility to study whether there really is an optimal position or area within the uterus for implantation. Once this area is known, endometrial biopsies in non transfer cycles will help us understand more of the dynamics of the endometrium.

Besides a change in transfer technique clinicians could also try to influence the implantation process by altering or adding medication during IVF treatment[18]. This medication might be directed to improve the quality of the uterine environment or endometrial quality. Increasing the flow of the uterine arteries

could theoretically lead to a more optimal condition of the endometrium[24], creating a 'soft bed' or a 'warm welcome' for an embryo entering the uterine cavity. Aspirin with its qualities of vasodilatation seemed to be a promising candidate[25]. In our aspirin study, we specifically analysed the pulsatility index of the uterine arteries as an indicator for the uterine blood flow. We found that administration of aspirin during IVF treatment did not improve the flow in the uterine arteries and that it also did not improve pregnancy rates[26]. In line with our aspirin trial, others authors have confirmed our findings by demonstrating that aspirin has no ability to improve pregnancy rates[27-29]. Meta-analyses still do not agree on the overall result of all aspirin studies[30-33]: does it or does it not have an effect on pregnancy rates in IVF? To solve this problem once and for all, we suggest that authors of aspirin trials keep their data available for a meta-analysis using the individual patient data.

Of special interest were the results of the follow up of our aspirin study, in which we found less hypertensive complications in the aspirin treated group. The effect of aspirin treatment may therefore not be represented in increasing the chance of implantation, but may be hidden in the quality of the implantation. This potential of pre-conceptional aspirin treatment in minimizing chances of hypertensive pregnancy complications should be further investigated in large randomized placebo controlled trials before any final conclusions can be drawn.

Future recommendations

The process of human embryo implantation is difficult to study in vivo, since it is hard to time the moment and it is a precious vulnerable biological process. The first part of the problem can be overcome by using an IVF setting in which the moment can be predicted. The fact that this is a precious and vulnerable process restricts us to indirect measurements and analyses. Therefore, it would be of great value to generate an in vitro model for embryo implantation to study the embryo-endometrial dialogue more directly and to compare single and multiple implantations.

The contribution of the endometrial component in the different potential of single and multiple implantations for continuation of pregnancy should be further analysed by comparing some of the known implantation factors such as VEGF and glycodeline-A. This could help to profile endometrial receptivity for embryo implantation and pregnancy continuation.

To gain more insight in the embryonic factors that determine the potential of an embryo for implantation, embryos are now analysed for their metabolomic profile. This profile will further distinguish the developmental potential of an embryo and together with the morphologic characteristics provide a new possibility to select the perfect embryo.

As we discovered from our studies on the ultrasonographic embryo transfer there is need for a more precise technique to transfer embryos. This calls for development of a transfer device that is able to transfer the embryo at an exact location in the uterus at a controlled speed. This will allow more precise embryo placement and therefore create a possibility to analyse what location in the uterus is the most optimal for embryo placement. Once this location is identified this could open up possibilities for histological comparison.
Finally, our findings from the follow up of the pregnant women in the aspirin trial calls for a large randomised controlled trial designed to analyse the potential effect of pre-conceptional aspirin administration to reduce hypertensive pregnancy complications in IVF patients.

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Summary

Summary

Chapter 1 contains the outline of the thesis.

In **chapter 2** we evaluate the accuracy of a single hCG determination to optimise the cut off values of the hCG-levels after embryo transfer. We found that a single serum hCG determination only 14 or 15 days after oocyte retrieval is sufficient for discrimination between viable and non-viable pregnancies. The level of this pregnancy hormone reflects the strength and potential of the implantation and provides patients with some essential perspective in a very early stage of pregnancy.

In **chapter 3** we demonstrated that the continuation of pregnancy is dependent on the combination of genetic and developmental potential of embryos and an optimal uterine milieu, while the occurrence of multiple implantation after DET in IVF is predominantly dependent on embryo quality.

Chapter 4 demonstrates that even though multiple ovulation has a hereditary basis, it is very unlikely that multiple implantation also has a hereditary basis itself. The findings of this study underline that multiple implantation after DET in IVF is mainly determined by the quality (and therefore potential) of the transfered embryos.

In **chapter 5** we studied the value of monitoring of the embryo transfer by ultrasound in comparison with a transfer technique that positions the catheter based on previous uterine length measurement. Although ultrasonographic monitoring of the transfer provides both patient and doctor with visual feedback of the transfer, it did not help to improve pregnancy rates.

With the visual feedback from the ultrasonographically monitored embryo transfer we demonstrated in **chapter 6** that there is a relation to the location of the transfer air bubble after embryo transfer to the clinical outcome of the IVF treatment. With the air bubbles as markers for the content of the transfer catheter we found that pregnancy rates were higher when the air bubbles ended in the upper half of the thick endometrial plate.

By repeating the ultrasound fifteen minutes after the ultrasonographically monitored embryo transfer we demonstrated in **chapter 7** that the final position of transfer content is not sensitive to immediate ambulation after embryo transfer. Changes in position of the transfer content in both groups of patients (immediate ambulation or bedrest after transfer) were directed in the same direction: towards the fundus.

Chapter 8 is an analysis of the effect of aspirin on pregnancy rates after IVF and on the Pulsatility Index of the uterine arteries throughout the treatment and first trimester of pregnancy. This prospective double blind placebo controlled randomised trial shows that use of aspirin during IVF treatment does neither influence the pregnancy rates nor the Pulsatility Index of the uterine arteries.

The follow up of the patients who became pregnant in the aspirin study was reported in **chapter 9**. From analysing the further course of their pregnancies we found that the pregnant patients from the aspirin treated group have less hypertensive pregnancy complications such as hypertension, pre-eclampsia and HELLP-syndrome.

Chapter 10 contains a general discussion of the results in this thesis and suggests future strategies for further research to unravel more of the secrets of embryo implantation.

Nederlandse samenvatting

Hoofdstuk 1 is een uiteenzetting van de achtergrond van dit onderzoek en het kader waarbinnen dit tot stand is gekomen.

In **hoofdstuk 2** evalueren we de accuraatheid van een enkele hCG bepaling om de cut-off waardes van hCG-levels na embryo transfer te optimaliseren. We toonden aan date en enkele serum hCG bepaling al op de 14e of 15e dag na IVF punctie toereikend is om een onderscheid te maken tussen levensvatbare en niet levensvatbare zwangerschappen. De hoogte van dit zwangerschapshormoon reflecteert de kracht en het potentieel van de implantatie en het voorziet patiënten van enig essentieel perspectief in een zeer vroege stadium van de zwangerschap.

De bevindingen in **hoofdstuk 3** laten zien dat het voortzetten van een zwangerschap afhankelijk is van de combinatie van genetisch en ontwikkelingspotentieel van embryo's met een optimaal uterien milieu, terwijl het optreden van multiple implantatie na DET in IVF voornamelijk van embryo kwaliteit afhangt.

Hoofdstuk 4 demonstreert dat ondanks dat er een erfelijke basis voor multiple ovulatie bestaat, het erg onwaarschijnlijk is dat multiple implantatie zelf ook een erfelijke basis heeft. De resultaten van deze studie benadrukken dat multiple implantatie na DET in IVF voornamelijk bepaald wordt door de kwaliteit (en daarmee het potentieel) van de embryo's die bij transfer in de baarmoeder geplaatst worden.

Hoofdstuk 5 bestudeert de waarde van echoscopische monitoring van de embryo transfer in vergelijking met een transfer techniek die de catheter positioneert op basis van eerdere metingen van de lengte van het cavum uteri. Hoewel echoscopische monitoring van de embryo transfer zowel patiënt als dokter voorziet van visuele feedback van transfer, zorgt het er niet voor dat de zwangerschapspercentages verbeteren.

Met behulp van de visuele feedback die verkregen werd van de echogeleide embryo transfer, toonden we in **hoofdstuk 6** aan dat er een relatie bestaat tussen the positie van de luchtbellen na de embryo transfer en de klinische uitkomst van de IVF behandelingen. Met de luchtbellen als markers voor de inhoud van de transfer catheter lieten we zien dat zwangerschapspercentages hoger waren wanneer de luchtbellen in de bovenste helft van de dikke endometrium plaat terecht kwamen.

Door de echo te herhalen vijftien minuten na de echogeleide embryo transfer, lieten we in **hoofdstuk 7** zien dat de uiteindelijke positie van de catheter inhoud niet gevoelig is voor directe mobilisatie na de embryo transfer. Veranderingen in de positie van de catheter inhoud waren in beide groepen (directe mobilisatie of bedrust) gericht in dezelfde richting: richting fundus.

Hoofdstuk 8 is een analyse van het effect van aspirine op zwangerschapspercentages na IVF behandeling en op de Pulsatility Index van de baarmoederlijke slagaders gedurende de gehele behandeling en eerste trimester van zwangerschap. Deze prospectieve dubbel blinde placebo gecontroleerde gerandomiseerde trial toont aan dat het gebruik van aspirine gedurende de IVF behandeling geen invloed heeft op de zwangerschapspercentages, maar ook niet op de Pulsatility Index van de baarmoederlijke slagaders.

De follow-up van patiënten die zwanger werden in de aspirine studie wordt gerapporteerd in **hoofdstuk 9**. Door het verdere beloop van hun zwangerschappen te analyseren vonden we dat de zwangere patiënten in de met aspirine behandelde groep minder vaak hypertensie gerelateerde zwangerschapscomplicaties, zoals zwangerschapshypertensie, pre-eclampsie en HELLP-syndroom, ontwikkelden.

Hoofdstuk 10 is een algemene beschouwing van de bevindingen in dit proefschrift en komt met suggesties voor toekomstig onderzoek om het proces van embryo implantatie verder in kaart te brengen.

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