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# Epidemiological analysis of important factors for natural cycles of poor responder women

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## Abstract

Since the first tube baby was born in 1978, *in vitro* fertilization (IVF) treatment offered high pregnancy rates due to improvements in oocyte punction and culture techniques, sperm preparation and oocyte retrieval methods and production of new recombinant stimulating hormones of the ovarian production. However, different disadvantages such as ovarian hyperstimulation, multiple pregnancies, premature birth, expensive and not without risk stimulating protocols, are often involved in IVF cycles. Our purpose was to assess the efficacy of IVF in natural cycles, as an alternative treatment in women with a poor ovarian response in gonadotropin stimulation and to explore the relationship

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between the important factors that control the natural cycle of women and the positive or negative β-human chorionic gonadotropin (β-HCG).

Twenty six women with regular menstrual were offered a total of 37 natural cycles of treatment. One woman had four natural cycles, two women had 3 natural cycles each and 4 women had 2. The women treated were from 29 to 46 years old and their mean age was 37,6±4,2 years old. LH surge was observed in 14 cycles (37,8%). In 20 cycles (54%) one oocyte was retrieved and in 2 cycles (5,4%) two oocytes were retrieved. The mean follicle diameter was 17,4±0,8mm and the mean endometrial thickness was 8,5±1,2mm of the day of hCG injection. From the 24 oocytes retrieved, 20 of them were in metaphase II stage (83,3%) and the fertilization rate after ICSI was 70% (14/20). All the embryos resulted from the fertilized oocytes were transferred after 2 or 3 days of incubation. The Body Mass Index (BMI) values and the smoking were also estimated as parameters of the life style of the women participated in the study. All statistical analysis was achieved by using $x^2$-test. Modeling was achieved by applying logistic regression methodology.

Embryo transfer was performed in 14 patients (37,8% per cycle, 70% per MII oocytes). Three patients were pregnant (8,1% per ocyte retrieval and 21,4% per embryo transfer). The quality of embryos transferred was grade 1 (no fragmentation) in 3 cases (21,4% per total number of embryos), grade 2 (<15% fragmentation) in 9 cases (64,3%) and grade 3 (>15% fragmentation) in 2 cases (14,3%). The three pregnancies resulted from one grade 1 four-cell embryo; one resulted from grade 2 five-cell embryo and one from a grade 3 six-cell embryo. The comparison between the βHCG and the other variables suggests that important factors are the thickness of the endometrium (p=0,028) and the follicle diameter (p=0,049), for 95% significant level. In our experimental work, the investigation of the variables after using the logistic regression modeling, suggests that the important factors which were estimated satisfactory for the positive or

negative βHCG, are the endometrium thickness (OR=18,74 times) and the embryo grade (OR=5,48 times) with estimated parameter values.

The natural cycle is a simple and effective method in the field of the assisted reproduction, especially for poor-responder women in gonadotropin stimulation. Furthermore, the final proposed statistical model explains satisfactory the 89.2% of the original data of our study.

**Keywords:** Natural cycle, poor responder women, pregnancy outcome, statistical modelling, logistic regression

# 1 Introduction

Although the first successful pregnancy and live birth from in vitro fertilization (IVF) resulted from an unstimulated natural menstrual cycle [1], the natural cycle IVF treatment was soon abandoned and replaced by ovarian stimulation with exogenous FSH in order to produce more oocytes and consequently, more embryos available for transfer. This fact resulted in fewer abandoned cycles and higher pregnancy rates, especially when down-regulation with gonadotropin-releasing hormone analogues prior to ovarian stimulation is applicable [2]. IVF treatment offered high pregnancy rates due to improvements in oocyte punction and culture techniques, sperm preparation and oocyte retrieval methods and production of new recombinant stimulating hormones of the ovarian production. However, the consequences of ovarian stimulation in IVF are numerous: a higher number of multiple pregnancies [3], ovarian hyperstimulation syndrome [4], a decrease in endometrial receptivity [5], a possible increased risk of developing ovarian cancer [6], legal and ethical problems about surplus embryos and an increase in costs [7]. The long-term side effects of ovarian stimulation with protocols involving a GnRH agonist or antagonist in combination with gonadotropins are also largely unknown. These problems lead us to re-evaluate natural cycle IVF (nIVF), although other problems arise in this case. Such problems include an increased risk of cancellation of the cycle due to a luteinizing hormone (LH) surge [8], poor follicle development [9], absence of oocyte, no fertilization or polyspermic fertilization with no embryo transfer [10] and finally, a lower pregnancy rate per started cycle versus stimulated IVF cycle [11]. It has been suggested that nIVF is best applied in women with tubal factor infertility [12]. Furthermore, the method of intracytoplasmic sperm injection (ICSI) in natural cycles is widely applied among patients with severe oligozoospermia [13]. The main purpose behind this study was the statistical investigation of the factors that control the efficacy of the natural cycle in women with a poor ovarian response in gonadotropin stimulation as well the introduction of a pilot model for the success of this type of assisted reproduction.

## 2 Methods

Twenty-six women with a regular menstrual cycle, but a low response in ovarian stimulation with gonadotropins, were offered a total of 37 natural cycles of treatment. One woman had four natural cycles, two women had three natural cycles each and four women had two. The women treated were from 29 to 46 years old (mean age: 37,6±4,2 years old). All couples participating in the study signed an informed consent.

On day 2, a baseline ultrasound scan excluded ovarian cysts and a measurement of baseline follicle stimulating hormone (FSH) value showed the number of women who were excluded from the study (FSH>10U/l) [8], because of the increased risk of failing to collect an oocyte in a natural cycle [14]. Cycle monitoring was performed with ultrasound, starting on day 8 or 10, depending on the length of the cycle. When the leading follicle reached a diameter >13mm, patients came in daily for an ultrasound, oestradiol and luteinizing hormone (LH) measurements. When the diameter of the leading follicle was approximately 18mm and the morning serum LH value less than 15U/l [15], the timing of oocyte retrieval was done by ovulation triggering with 250UI HCG (Ovitrelle, Serono Hellas S.A., Athens, Greece). The interval between HCG injection and oocyte retrieval ranged from 34 to 36 hours. In some cases, when the morning serum LH value was elevated, the oocyte retrieval was programmed for the next morning.

The oocyte punctions were performed on any day of the week, using a double lumen aspiration needle and a vaginal ultrasound, under a slight general anesthesia. Oocytes were cultured in G-Fert (GIII Vitrolife Sweden AB, Sweden).

For the insemination of all the oocytes retrieved, the method of micromanipulation (ICSI) was applied, using motile spermatozoa prepared by the technique of the two-density gradients centrifugation (90%, 50%, Pure Sperm, Nydacon, Sweden). The coronal cells were carefully removed by use of a series of finely drawn glass Pasteur pipettes after exposure to 80IU/ml hyalase (Sigma-Aldrich, Missouri, USA) for 30 seconds, in Hepes-buffered HTF (Irvine Scientific, Santa Ana, USA). All incubations were realised in G1 culture medium (GIII Vitrolife Sweden AB, Sweden) a humidified gas mixture of 6% $CO_2$ and 5% $O_2$ at 37$^o$C. Fertilization was assessed the next morning and in case of cleavage, the transfer of a single embryo was performed 48 hours or 72 hours after oocyte retrieval in Glue medium (GIII Vitrolife Sweden AB, Sweden), using a Wallace catheter. The luteal phase was supported with 2mg ethynyl-oestradiol (Cyclacur, Schering Hellas S.A., Athens, Greece) and 3x100mg natural progesterone (Utrogestan, Laboratories BESINS INTERNATIONAL S.A., Paris, France). The pregnancy test was performed on the 15$^{th}$ day after oocyte retrieval, in all patients who underwent an embryo transfer. The pregnancy was confirmed by ultrasonic evidence of a gestational sac.

Concerning the creation of the statistical mode, the parameters involved in the analysis were: β-hCG, age of women. LH surge, number of oocyte, number of mature oocytes (metaphase II oocytes), number of fertilized oocytes, grade of embryos, endometrial thickness, grade of transfer and body mass index (BMI=weight/height$^2$). The last parameter was estimated as an index of the life style of the women participating in the study.

The main goal of the study was to find the best fitting and most applicable reasonable model to describe the relationship between an outcome (dependent variable) and a set of independent variables (predictors). The method (logistic regression) [16, 17] would be used in case where the outcome variable is binary or dichotomous. In our case, it is of interest to explore the relationship between the important factors that control the natural cycle of the women and the positive or negative β-human chorionic gonadotropin (β-HCG) in this investigated population. In our experimental work, the variables that were chosen for investigation were the significant ones achieved from the $x^2$-testing.

## 3 Results

In order to obtain one oocyte from each of 26 different patients, 37 natural cycles of treatment were needed. LH surge was observed in 14 cycles (37,8%). In 20 cycles (54%), one oocyte was retrieved and in 2 cycles (5,4%), two oocytes were retrieved. The mean follicle diameter was 17,4±0,8mm and the mean endometrial thickness was 8,5±1,2mm on the day of hCG injection. No difference in the oestradiol concentrations were observed on the day of the HCG trigger between the cycles in which one oocyte was collected (195-395pmol/l) or was not collected (180-403pmol/l). From the total of 24 oocytes collected, 20 of them were in metaphase II stage (83,3%), two of them were in metaphase I stage (8,33%), one of them was a germinal vesicle oocyte (4,16%) and one oocyte was lysed (4,16%). The fertilization rate after ICSI was 70% (14 out of 20 metaphase II oocytes).

Table 1: Results of 42 natural ICSI cycles

| | |
|---|---|
| No of cycles | 42 |
| Premature ovulation | 5 |
| Oocyte retrieval: | 37 |
| Successful | 22 (59,46%) (20 cycles with 1 oocyte retrieved and 1 cycle with 2 oocytes) |
| Not successful | 15 (40,54%) |
| Oocytes after denudation: | (% per oocyte retrieval) |
| MII oocytes | 20 (83,3%) |
| MI oocytes | 2 (8,33%) |
| GV oocytes | 1 (4,16%) |
| other | 1 x lysed |
| Result of ICSI: | (% per metaphase II oocyte) |
| Fertilisation (2 pronuclei) | 14 (70%) |
| No fertilisation | 6 (30%) |
| Nr of ET | 14 |
| Result of ET: | (% per oocyte retrieval,%per ET) |
| Pregnancy | 3 |

Embryo transfer was performed in 14 patients (37,8% per oocyte retrieval, 70% per metaphase II oocyte), 2 or 3 days after the oocyte retrieval. Three patients were pregnant (21,4% per embryo transfer) and the live birth rate was 8,1% per performed cycle (3 of 37 cycles). The quality of embryos transferred were grade 1 (no fragmentation) in 3 cases (21,4% per total number of embryos), grade 2 (<15% fragmentation) in 9 cases (64,3%) and grade 3 (>15% fragmentation) in 2 cases (14,3%). One pregnancy resulted from grade 1 four-cell embryo; one resulted from grade 2 five-cell embryos and one from a grade 3 six-cell embryos. All pregnancies resulted in the term birth of a healthy child (Table 1). Tables 2 and 3 show that βHCG is positive in patients with normal BMI (20-27,9 kg/m$^2$) or underweight (<20 kg/m$^2$) patients with very good or good grade of embryos, but not in obese (≥28 kg/m$^2$) patients with excellent embryos.

Table 2: Correlation between embryo quality and BMI

| Embryo Quality | Normal BMI | Underweight | Obese |
|---|---|---|---|
| Excellent (no fragmentation) | 0 | 1 | 2 |
| Very good (fragm. <15%) | 9 | 0 | 0 |
| Good (fragm. >15%) | 1 | 1 | 0 |

Table 3: The relationship between βHCG and BMI

| βHCG | Normal BMI | Underweight | Obese |
|---|---|---|---|
| Negative | 8 | 1 | 2 |
| Positive | 2 | 1 | 0 |

The treated women were from 29 to 46 years old, with mean age 37,86 years and 95% confidence interval of the mean between 33,62-42,1 years. The mean thickness of the endometrium was 8,35mm and 95% confidence interval of the mean was between 7,31-9,38mm. Concerning the oocyte diameter before the retrieval, its mean value was 17,4mm and 95% confidence interval of the mean was between 16,7-18,09mm. The mean value of BMI was 23,5kg/m$^2$ and 95% confidence interval of the mean was between 18,85-28,33kg/m$^2$ (Table 4).

Table 4: Descriptive statistics for quantitative factors

| Factors | Mean value | 95% conf. interval of the mean | Range |
|---|---|---|---|
| Age (years) | 37,86 | 33,62-42,1 | 29-46 |
| Endometrium thickness (mm) | 8,35 | 7,31-9,38 | 6,8-11 |
| Oocyte diameter (mm) | 17,4 | 16,70-18,09 | 16-19 |
| BMI (kg/m$^2$) | 23,5 | 18,85-28,33 | 18,7-44,1 |

The comparison between the βHCG and the other variables suggests that important factors are: 1. the thickness of the endometrium (p=0,028); 2. the grade of embryos (p=0,024); the follicle diameter (p=0,049) for 95% significant level (Table 5).

Table 5: Comparison of different clinical and embryological parameters

| Factors | $x^2$-test | p-value | Status |
|---|---|---|---|
| βHCG/endometrium thickness | 4,85 | 0,028 | Significance (95%) |
| βHCG/embryo grade | 9,41 | 0,024 | Significance (95%) |
| βHCG/oocyte diameter | 1,71 | 0,049 | Significance (95%) |

In our experimental work, the investigation of the variables after using the logistic regression modeling, suggests that the important factors which were estimated satisfactory for the presence or absence of βHCG, are the endometrium thickness and the embryo grade with estimated parameter values (Table 6). The final proposed model (Eq. 1) is:

$$\ln\left[\frac{p}{1-p}\right] = -11.947 + 2.931 * \text{endomytrium thickness} + 1.702 * \text{embryo grade} \quad (1)$$

Table 6: Investigation of the variables after using the logistic regression modeling

| | B | S.E. | p | Exp (B) |
|---|---|---|---|---|
| Endometrium thickness | 2,931 | 1,659 | 0,047 | 18,74 |
| Embryo grade | 1,702 | 1,015 | 0,027 | 5,48 |
| Constant | -11,947 | 5,461 | 0,029 | 0,000 |

The above model explains satisfactory the 89,2% of the original data as it is shown in Table 7. As a result of the pilot modeling, the positive βHCG in natural cycles of poor responders patients depends 5,48 times (OR=5.84) on the embryo quality and 18,74 times (OR=18.74) on the endometrium thickness, both statistically significant (p<0,05).

Table 7: Classification Table

| Observed | Predicted | | |
|---|---|---|---|
| | Negative βHCG | Positive βhCG | % Correction |
| Negative βHCG | 32 | 2 | 94,1 |
| Positive βHCG | 2 | 1 | 33,3 |
| Overall percentage | 34 | 3 | 89,2 |

A receiver operating characteristic (ROC), or simply ROC curve, is a graphical plot of the sensitivity vs. (1 - specificity) for a binary classifier system as its discrimination threshold is varied. The ROC can also be represented equivalently by plotting the fraction of true positives (TPR = true positive rate) vs. the fraction of false positives (FPR = false positive rate). The accuracy of the test depends on how well the test separates the group being tested into those with and without the variable is examined. Accuracy is measured by the area under the ROC curve. Figure 1 illustrates the graphical presentation of the sensitivity using ROC curves for the endometrium thickness and the embryo grade considering the presence of βHCG diagnosis with accuracy 0.765 (endometrium thickness) and 0.848 (embryo grade) (Figure 1).

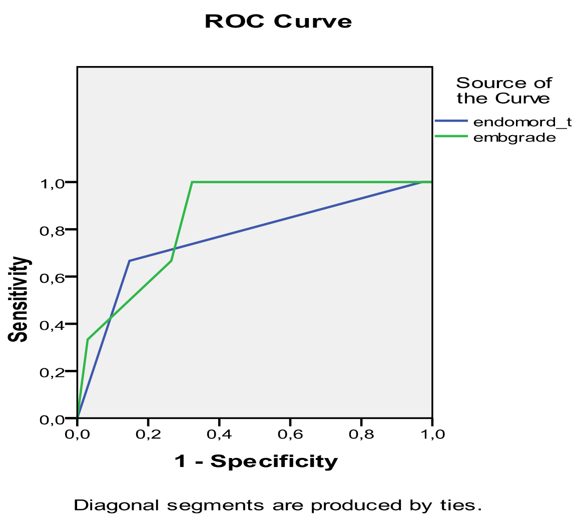


Figure 1: ROC curve for βHCG versus endometrium thickness and embryo grade: blue line: endometrium thickness; green line: embryo grade

## 4 Conclusions

The analysis of the results of this study indicates that the application of in vitro fertilization (IVF) in natural cycles is an effective method of treatment for ovulatory but poor responders' women in gonadotropin stimulation of their ovaries. In different studies, natural cycle IVF has been proposed as a valuable alternative to stimulated IVF in poor responders [18, 19]. Ziebe *et al.* [20] in a retrospective analysis, suggest that the administration does not concern the morphology of the early stages of embryonic development. Furthermore, no differences observed between stimulated cycles and natural cycles, concerning the cleavage rate of the oocytes and the early cleabage stage morphology assessment of the embryos. In the same study, it is suggested that the only advantage of the hormonal stimulation of the ovarian activity increases the number of oocytes retrieved resulting in achieving more embryos to select from at the time of transfer. On the other hand, the hormonal environment in the natural cycle is theoretically the optimal one for the maturing follicle and oocyte. Kaneko *et al.* [21] showed that controlled ovarian stimulation participates in the apoptosis of granulosa cells.

IVF is often linked with embryo heterogeneity both in terms of morphology [22, 23] and chromosomal constitution [24]. Van Blerkom and Davis [25] studied the repeated ovarian stimulation in mice which results in a significant increase in the frequency of spindle defects and consequently, in chromosomal errors during the ovarian stimulation. Some studies show diminished endometrial receptivity as a result of increased levels of steroid hormones after ovarian stimulation for IVF [26, 27]. On the other hand, steroid hormones levels in unstimulated IVF cycles are physiological, which theoretically leads to better endometrial receptivity, but the lower implantation rates per embryo may be due to the fact that no embryo selection is possible in natural cycles. In our study, we found a big fertilization rate (70%) in retrieved oocytes after ICSI in agreement with the study of Lukassen *et al.* [13] and our cumulative live birth rates after four cycles of treatment is 32,4%, which is in agreement with those presented in other studies [28, 29, 30].

Natural cycle IVF has many potential advantages. Firstly, it is a relatively easy procedure mainly for the patient and for the laboratory staff as well [12]. There are fewer visits of the patients to the IVF centre and the oocyte retrieval is considerably shortened and less painful. Secondly, the ovarian hyperstimulation syndrome (OHSS) is avoided in natural cycles (30). Thirdly, the patients do not have a risk for multiple pregnancies, as in natural cycles IVF; the transfer is done with one embryo. Although, we have to mention that multiple pregnancies is a consequence of embryo transfer policy and not of ovarian stimulation in itself. However, elective single embryo transfer is applied for selected and good-prognosis patients and most IVF centers do not participate in this standard policy [31].

Fourthly, the natural cycle IVF is cheaper than stimulated IVF [27]. The avoidance of expensive drugs and the reduced number of ultrasound scans makes the treatment less expensive and stressful for the patient. The prevention of OHSS also leads to a reduction in costs as the hospitalization is avoided [12]. Fifthly, it is sure that the application of a natural cycle IVF means that no further frozen embryos are available for a second "chance" for the couple. Although, some IVF units present high pregnancy rates with frozen embryos transfer, the average live birth rate in the UK is 12,3%, which is near of that of repeated natural cycle treatments [29].

An important disadvantage of natural-cycle treatments is the high cancellation rate because of abnormal follicular development or premature LH surge, although the regular menstrual cycle of the treated women. In our study, the cancellation rate was 45,9% (17 of 37 cycles), which is similar of that in the study of Lukassen *et al.* [13].

Concerning the embryo quality (number of blastomeres and grade of fragmentation), it is possible that during the follicular development, the nature selects a relatively good embryo to ovulate. Despite these expectations, only 3 of 14 embryos (21,4%) were of excellent quality in this study (no fragmentation), in agreement with Lukassen *et al.* [13]. Only one of these embryos implanted and led to the birth of a healthy baby. The second pregnancy was obtained by an embryo of grade 2 quality (fragments <15%) and the third one, by an embryo of grade 3 quality (fragments >15%).

Consequently, if the best oocyte is selected during natural follicular development, this face is not found in our study, in agreement with the equivalent study of Lukassen *et al.* [13]. Concerning the influence of body mass index (BMI) on IVF success (natural or stimulated), the controversy that exists among different studies [32, 33, 34] is also verified in our study: the positive β-HCG concerned the normal and underweight patients and not the obese, in agreement with the studies of Jones *et al.* [33] and Loveland *et al.* [34]. It is sure that the small number of cases of our study led us not to conclude with safety about the participation of the BMI in natural cycles outcomes. In general, it seems that body composition exerts a greater influence on IVF success and leptin plays an important role as humoral signal of energy stores to the central nervous system and reproductive tissues [35].

Concerning the main goal of this study, we tried to create a pilot statistical model, in order to investigate the important factors that affect the natural cycle of the women who participated in this work. To achieve this purpose, statistical methods were implemented firstly to investigate significant differences between clinical and embryological characteristics of the natural cycle and secondly to model these parameters (using logistic regression technique) in order to predict the pregnancy. From the statistical analysis, we concluded that follicle's diameter, the endometrium thickness, significantly achievement of the pregnancy in natural cycles. Especially, last two parameters were judged satisfactory (89,2%) for a positive βHCG.

In conclusion our study suggests that natural cycle could be a proposed alternative method in comparison with stimulated cycles, in poor responders women in gonadotropin ovarian stimulation. Furthermore, natural cycle is a relatively easy, cost effective and realistic option to achieve a satisfactory pregnancy rate (8,1% per oocyte retrieval, 21,4% per embryo transfer). Extrapolation of 8,1% live birth rate after one natural cycle yields a cumulative live birth rate after four natural cycles of 32,4%.

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