Maternal hCG concentrations in early IVF pregnancies: associations with number of cells in the Day 2 embryo and oocytes retrieved

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STUDY QUESTION: Do number of cells in the transferred cleavage stage embryo and number of oocytes retrieved for IVF influence maternal hCG concentrations in early pregnancies?

SUMMARY ANSWER: Compared with transfer of a 2-cell embryo, transfer of a 4-cell embryo results in higher hCG concentrations on Day 12 after transfer, and more than 20 oocytes retrieved were associated with low hCG concentrations.

WHAT IS KNOWN ALREADY: Maternal hCG concentration in very early pregnancy varies considerably among women, but is likely to be an indicator of time since implantation of the embryo into the endometrium, in addition to number and function of trophoblast cells.

STUDY DESIGN, SIZE, DURATION: We followed 1047 pregnancies after IVF/ICSI from oocyte retrieval until Day 12 after embryo transfer. Women were recruited in Norway during the years 2005–2013.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Successful pregnancies after transfer of one single embryo that had been cultured for 2 days were included. Maternal hCG was quantified on Day 12 after embryo transfer by chemiluminescence immunoassay, which measures intact hCG and the free β-hCG chain. Information on a successful pregnancy, defined as birth after >16 weeks, was obtained by linkage to the Medical Birth Registry of Norway.

MAIN RESULTS AND THE ROLE OF CHANCE: Transfer of a 4-cell embryo resulted in higher maternal hCG concentrations compared with transfer of a 2-cell embryo (134.8 versus 87.8 IU/l, \(P < 0.05\)). A high number of oocytes retrieved (>20) was associated with low hCG concentrations \((P < 0.05)\).

LIMITATIONS, REASONS FOR CAUTION: The factors studied explain a limited part of the total variation of hCG concentrations in early pregnancy. Although embryo transfer was performed at the same time after fertilization, we do not know the exact time of implantation. A further limitation to our study is that the number of pregnancies after transfer of a 2-cell embryo was small (27 cases).

WIDER IMPLICATIONS OF THE FINDINGS: Number of cells in the transferred embryo and number of oocytes retrieved may influence the conditions and timing for embryo implantation in different ways and thereby influence maternal hCG concentrations. Such knowledge may be important for interpretation of hCG concentrations in early pregnancy.

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Key words: hCG / IVF / embryo / oocytes / pregnancy
Introduction

hCG is a hormone that is mainly produced during pregnancy, and hCG of pregnancy is synthesized in trophoblast cells. Already at the preimplantation stage, the embryo secretes hCG to prepare the endometrium for implantation (Licht et al., 1998, 2001; Herrell et al., 2003). Shortly after implantation of the embryo into the endometrium, hCG may be detected in maternal serum or in the urine. In a normal pregnancy, maternal hCG concentration increases rapidly, shortly after embryo implantation (Bouduelle et al., 1988; Wilcox et al., 1988).

A primary function of hCG is maintenance of the corpus luteum during the early weeks of pregnancy. However, hCG and its isoforms also have several other biological functions that are important for the developing embryo, as hCG induces trophoblast invasion and angiogenesis, both vital for placental development. Following a successful implantation, hCG also stimulates immunotolerance during pregnancy, uterine and fetal growth and suppression of uterine muscle contraction (Cole, 2010; Tsampalas et al., 2010).

There is a large inter-individual variation of maternal serum concentrations of hCG in early pregnancy (Ooi et al., 1989; Ertezid et al., 2000). The causes of such variation remain largely unknown, but in vital pregnancies, low hCG concentrations shortly after implantation have been associated with maternal obesity and high maternal age (Eskild et al., 2012; Haavaldsen et al., 2014). The consequences of low hCG concentrations may be increased risk for pre-eclampsia later in pregnancy (Åsvold et al., 2014a,b).

Better knowledge about determinant factors for hCG concentrations in early pregnancy may increase our understanding about the regulation of early pregnancy development, and thereby identify factors that could cause adverse pregnancy outcomes.

Maternal hCG concentrations in early pregnancy are likely to be linked to the number and function of trophoblast cells. In pregnancies after IVF, the number of cells in a transferred cleavage stage embryo typically varies between two and eight cells, corresponding 2–3 days in culture after oocyte retrieval. The number of cells in a cleavage stage embryo at a certain time in culture could indicate mitotic cleavage rate in that particular embryo. In embryos with high cell cleavage rate, it is likely that hatching and implantation of the embryo occur earlier as compared with embryos with a slow cleavage rate. In a recent study, number of cells in the cleavage stage embryo was associated with a successful pregnancy (Rhenman et al., 2015). We are not aware of any previous studies on the relation between number of cells in a cleavage stage embryo and maternal hCG concentrations.

Successful implantation of the embryo, with subsequent invasion and proliferation of trophoblast cells, depends on the receptivity of the endometrium. Thus, factors that influence the receptivity of the endometrium may also influence hCG concentrations in early pregnancy. In rodents, high estradiol (E2) concentrations have an adverse effect on uterine receptivity (Ertezid and Storeng, 2001). E2 is synthesized in follicles in the ovaries, and a high number of pre-ovulatory follicles may therefore indicate high serum E2 concentrations. Subsequent to final follicular maturation with hCG in IVF, several corpora lutea will be formed, synthesizing supraphysiological concentrations of both E2 and progesterone in the early luteal phase, including the implantation window. It is conceivable that a high number of oocytes retrieved in IVF can lead to embryo-endometrial asynchrony with subsequent suboptimal conditions for implantation of the embryo. Hence, the proliferation of trophoblast cells may also be suboptimal and the hCG concentrations may thereby be low.

We studied the associations of the number of cells in the transferred cleavage stage embryo and the number of oocytes retrieved in the IVF treatment cycle with maternal hCG concentrations on Day 12 after embryo transfer among 1047 successful pregnancies after IVF.

Materials and Methods

Study sample

At the Section for Reproductive Medicine, Oslo University Hospital, Rikshospitalet, Norway, during the years 2005–2013 there was a total of 2873 successful pregnancies after IVF in fresh cycles with or without ICSI. All oocytes used for treatment were autologous. Information about treatment success (birth after 16 weeks of gestation) was obtained through individual linkage to the Medical Birth Registry of Norway (Irgens, 2000), by using the unique person identification number given to all individuals with residency in Norway. We included successful pregnancies after transfer of one cleavage stage embryo, a total of 1305 pregnancies. Of these, we excluded 236 pregnancies for which maternal hCG concentrations were not measured on Day 12 after embryo transfer. Of the remaining pregnancies, we included only pregnancies after transfer of one embryo that had been cultured for 2 days and transferred on Day 2 after oocyte retrieval. Thus, we excluded 19 pregnancies after 3 days of embryo culture and 3 pregnancies with outlying values on duration of culture. The remaining 1047 pregnancies were included in our study.

Study factors

Information about the study factors was obtained from the electronic patient record at the Section for Reproductive Medicine, Oslo University Hospital, Rikshospitalet.

As outcome measure, we used hCG concentrations (IU/l) in a maternal serum sample drawn in the morning on Day 12 after embryo transfer (day 14 after oocyte retrieval). Serum hCG concentrations were quantified at the Department of Medical Biochemistry, Oslo University Hospital, Rikshospitalet by using an electro-chemiluminescence immunoassay method (Elecsys; Roche, Basel, Switzerland), which measures intact hCG and free β-hCG chain with a detection limit of 0.5 IU/ml. Control analyses at our hospital have shown a low within-series variation (coefficient of variation < 4%) and low variation over time (coefficient of variation < 5%). This is in agreement with the corresponding figures given by the manufacturer (Eskild et al., 2012).

Clinical and laboratory procedures

Our IVF treatment procedures have been described previously (Opøien et al., 2012). In short: a GnRH-agonist, nafarelin (Synarel, Pfizer, Barcelona, Puerto Rico) or buserelin nasal spray (Suprecur, Aventis Pharma, Frankfurt am Main, Germany) was used for pituitary down-regulation from the mid-luteal phase of the menstrual cycle preceding the treatment cycle. After pituitary down-regulation was achieved, stimulation with recombinant FSH was started (Gonal F, Serono, Aubonne, Switzerland or Puregon, Organon, Oss, the Netherlands). The initial daily FSH dosage was usually 150 IU s.c. in patients 35 years or younger and 225 IU in patients older than 35 years. A GnRH-antagonist stimulation protocol was used for some patients, and a total of 119 (11%) of the women received such treatment, starting with FSH stimulation on Day 2 or 3 of the menstrual cycle, and 5 days later, 25 microgram. GnRH antagonist (Orgalutran, Organon, Oss, the Netherlands) was given s.c. daily. The GnRH-antagonist treatment did not significantly influence maternal hCG concentrations and was therefore not included as a
formed the assessment as a routine part of the IVF treatment. Both GnRH analogues were administered until final follicle maturation with hCG. Oocyte retrieval was performed by using the vaginal ultrasonographical guided technique. Diploid fertilization was verified in the morning on the day after oocyte retrieval. The number of cells in the embryo was assessed by inverted light microscopy at 200–400× magnification in the morning of Day 2 after oocyte retrieval, corresponding to the day of embryo transfer (Van den Abbel et al., 1988). Trained embryologists at our department performed the assessment as a routine part of the IVF treatment.

Statistical analyses
For a description of the study sample as a whole, we calculated means and ranges of the values for continuous study factors, and proportions for categorical study factors. We estimated mean maternal hCG concentrations according to number of cells in the transferred embryo and the number of oocytes retrieved (in categories: ≤5, 6–10, 11–15, 16–20, 21–25 and >25). Differences in mean values were tested by analysis of variance. By applying linear regression analyses, we estimated crude and adjusted associations of number of cells in the embryo and number of oocytes retrieved (as a continuous variable) with hCG concentrations as unstandardized regression coefficients (B) with 95% confidence intervals. The unstandardized regression coefficient can be interpreted as changes in hCG concentrations by one unit change in number of cells in the embryo or number of oocytes retrieved. In additional analyses, we made adjustments for maternal BMI (kg/m²) (Eskild et al., 2012), age (in years) (Haavaldsen et al., 2014) and being a first time mother (yes/no). We repeated the analyses using log-transformed hCG concentrations as the outcome measure.

The statistical analyses were performed using the IBM Statistical Package for the Social Sciences version 21 for Windows (IBM Corp. Armonk, NY, USA). A value of $P < 0.05$ was considered significant.

Ethics
The use of clinical data for our study was approved by the Data Protection Officer at Oslo University Hospital, Rikshospitalet (License number 08/3438). The linkage between clinical data and the Medical Birth Registry of Norway was approved by the Regional Committee for Ethics in Medical Research (Reference number 2011/2465).

Results
Characteristics of the study sample are presented in Table I. On Day 12 after embryo transfer, mean maternal hCG concentrations were higher after transfer of a 4-cell compared with a 2-cell embryo (134.8 versus 87.8 IU/l, $P = 0.002$) (Table II). hCG concentrations after transfer of a 5-cell embryo ($n = 22$) seemed to deviate from an increasing trend, however, no significant difference from hCG concentrations after transfer of a 4-cell embryo was estimated ($P = 0.09$).

In pregnancies after retrieval of more than 20 oocytes, the mean hCG concentrations were significantly lower than after retrieval of fewer oocytes (114.0 IU/l (SD 57.8) versus 133.7 IU/l (SD 67.0, $P = 0.026$) (Table II).

In crude linear regression analyses, the estimated hCG concentrations increased by number of cells in the embryo, but decreased by number of oocytes retrieved (used as a continuous variable) (Table III, Fig. 1). After mutual adjustments, these factors remained significantly associated with hCG concentrations. Additional adjustment for maternal BMI, age and parity did not change the estimated associations notably (Table III). Also when log-transformed hCG concentrations were used as the dependent variable, the results remained almost unchanged (data not shown).

Discussion
In this study of 1047 successful pregnancies after IVF, the maternal hCG concentrations were higher 12 days after transfer of a 4-cell compared with a 2-cell embryo. Retrieval of a high number of oocytes (more than 20) in the index IVF cycle was associated with low hCG concentrations.

Strengths and limitations
In spontaneous pregnancies, the time from ovulation to implantation of the embryo may vary substantially (Wilcox et al., 1999; Jukic et al., 2011).

### Table I Study factors among 1047 singleton pregnancies after IVF/ICSI.

<table>
<thead>
<tr>
<th></th>
<th>Mean (SD)</th>
<th>Range</th>
<th>Proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cells in the</td>
<td>3.96 (0.36)</td>
<td>2–5</td>
<td></td>
</tr>
<tr>
<td>2 day embryo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of oocytes</td>
<td>10.34 (5.40)</td>
<td>1–34</td>
<td></td>
</tr>
<tr>
<td>retrieved</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hCG concentrations (IU/l)</td>
<td>132.91 (66.73)</td>
<td>1–588</td>
<td></td>
</tr>
<tr>
<td>on Day 12 after embryo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>transfer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.70 (3.92)</td>
<td>16.85–36.84</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>32.68 (3.37)</td>
<td>22–41</td>
<td></td>
</tr>
<tr>
<td>First time mother</td>
<td>69.3%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are mean (SD) and range.

### Table II Maternal hCG concentrations on Day 12 after embryo transfer, according to study factors among 1047 singleton pregnancies after IVF/ICSI.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>hCG (IU/l) Mean (SD)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells in the Day 2 embryo</td>
<td></td>
<td></td>
<td>ANOVA</td>
</tr>
<tr>
<td>2</td>
<td>27</td>
<td>87.8 (40.0)</td>
<td>32–201</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>131.1 (102.2)</td>
<td>32–349</td>
</tr>
<tr>
<td>4</td>
<td>990</td>
<td>134.8 (66.6)</td>
<td>1–588</td>
</tr>
<tr>
<td>5</td>
<td>22</td>
<td>106.2 (62.3)</td>
<td>47–278</td>
</tr>
<tr>
<td>Oocytes retrieved</td>
<td></td>
<td></td>
<td>ANOVA</td>
</tr>
<tr>
<td>≤5</td>
<td>197</td>
<td>138.3 (68.6)</td>
<td>24–339</td>
</tr>
<tr>
<td>6–10</td>
<td>381</td>
<td>130.9 (60.8)</td>
<td>26–374</td>
</tr>
<tr>
<td>11–15</td>
<td>295</td>
<td>134.3 (67.9)</td>
<td>1–411</td>
</tr>
<tr>
<td>16–20</td>
<td>122</td>
<td>136.6 (79.5)</td>
<td>34–588</td>
</tr>
<tr>
<td>21–25</td>
<td>42</td>
<td>118.0 (54.4)</td>
<td>45–312</td>
</tr>
<tr>
<td>&gt;25</td>
<td>10</td>
<td>81.6 (67.3)</td>
<td>32–262</td>
</tr>
</tbody>
</table>

Data are mean (SD) and range. ANOVA, analysis of variance.
In pregnancies after IVF, the time of fertilization of the oocyte is known; however, the exact time of implantation of the embryo is not known. In our study, all women had one embryo transferred after culture in vitro for 2 days, and hCG concentrations were measured in the morning on Day 12 after embryo transfer. hCG that was given to induce final follicle maturation has a half-life of 2.3 days, and could perhaps have affected results. However exogenously administered hCG from a single dose is cleared from the circulation after 14 days, that is, 2 days before we measured the serum level of hCG (Damewood et al., 1989). Thus, we have tried to standardize factors, other than our study factors, that could influence hCG concentrations. Maternal BMI (Eskild et al., 2012) and maternal age (Haavaldsen et al., 2014) have been associated with hCG concentrations, but adjustment for these factors and parity did not change our estimates. Two different fertilization techniques were used in our study, conventional IVF and ICSI. In a previous study (Gold et al., 2000), there were no differences in hCG concentrations in successful pregnancies after IVF versus ICSI. Therefore we made no adjustment for fertilization technique. Although number of cells in the embryo or number of oocytes retrieved may have been misclassified for some pregnancies, there is no reason to believe that possible misclassification has been differential according to subsequent hCG concentrations. Any misclassification tends to underestimate rather than overestimate an association.

The variance in hCG concentrations is large, and only 0.9% of the variance in our study could be explained by number of oocytes retrieved and number of cells in the embryo (data not shown). Thus, most of the variance in hCG concentrations is explained by other factors.

**Interpretation of findings**

Maternal hCG cannot be measured before implantation of the embryo into the endometrium. Thus, in very early pregnancy, time since implantation of the embryo is likely to be a determinant factor of maternal hCG concentrations. Also, the number and function of trophoblast cells in the implanted embryo may be closely linked to maternal hCG concentrations. Our findings may therefore suggest that the number of cells in the transferred embryo, and the number of oocytes retrieved from multiple ovarian follicles in the IVF cycle are linked to the timing of embryo implantation, to trophoblast proliferation or both. We are not aware of any other studies that have addressed the associations of number of cells in the embryo and number of oocytes retrieved in the IVF treatment cycle with maternal hCG concentrations.

In our study, the mean hCG concentrations 12 days after transfer of a 4-cell embryo were increased by 65% compared with a 2-cell embryo. If hCG concentrations reflect the number of functional trophoblast cells in the IVF cycle are linked to the timing of embryo implantation, to trophoblast proliferation or both. We are not aware of any other studies that have addressed the associations of number of cells in the embryo and number of oocytes retrieved in the IVF treatment cycle with maternal hCG concentrations.

In our study, the mean hCG concentrations 12 days after transfer of a 4-cell embryo were increased by 65% compared with a 2-cell embryo. If hCG concentrations reflect the number of functional trophoblast cells, our findings suggest that the relative difference in number of cells was larger at the time of embryo transfer than 12 days later. Thus, our finding is not consistent with a higher cell cleavage rate in a 4-cell embryo, but rather a somewhat delayed start of cleavage in the 2-cell embryo with subsequent later hatching and implantation. Thus, a transferred 2-cell embryo may have a similar or even higher proliferation rate than a 4-cell embryo in very early pregnancy. Mean doubling time from a

**Table III** Crude and adjusted associations of number of cells in the Day 2 embryo and number of oocytes retrieved with maternal hCG concentrations on Day 12 after single embryo transfer in 1047 successful pregnancies, as estimated by linear regression analyses.

<table>
<thead>
<tr>
<th></th>
<th>Crude unstandardized coefficient</th>
<th>95% CI</th>
<th>Adjusted unstandardized coefficient (1)</th>
<th>95% CI</th>
<th>Adjusted unstandardized coefficient (2)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cells in the embryo</td>
<td>13.6</td>
<td>2.4 to 24.8</td>
<td>14.6</td>
<td>3.4 to 25.8</td>
<td>15.8</td>
<td>4.7 to 26.965</td>
</tr>
<tr>
<td>Number of oocytes retrieved</td>
<td>−0.7</td>
<td>−1.4 to 0.05</td>
<td>−0.8</td>
<td>−0.2 to −0.02</td>
<td>−0.9</td>
<td>−1.6 to −0.13</td>
</tr>
</tbody>
</table>

All P-values are two-sided.

CI, confidence interval.

(1) Mutual adjustments for number of cells in the embryo and numbers of oocytes retrieved.

(2) Mutual adjustments for cells in the embryo, number of oocytes retrieved, age, BMI and being first time mother (yes or no).

**Figure 1** Number of oocytes retrieved and maternal hCG concentrations. The crude association of number of oocytes retrieved for IVF/ICSI with maternal hCG concentrations 12 days after transfer of a 2 day embryo, in 1047 successful pregnancies (linear regression, $P = 0.07$).
2-cell embryo to a blastocyst has been estimated to 16 h, but may vary considerably (Edwards et al., 1981). In our study, embryo development was evaluated at a fixed time interval after oocyte retrieval and fertilization. With time-lapse imaging of the embryo, a more dynamic evaluation of fertilization and early embryonic development is obtained which might possibly have influenced our results. However, in our laboratory, time-lapse imaging was introduced during the last years of our study period and only for evaluation of embryos after ICSI, therefore results from time-lapse imaging analysis were not included in the study.

Factors that influence the endometrium may also influence embryo implantation and thereby proliferation and invasion of trophoblast cells. It is likely that women with a high number of pre-ovulatory follicles in the ovaries in the index IVF cycle have high pre-ovulatory E2 concentrations, since E2 is synthesized in the follicles. It has been speculated that high E2 concentrations shortly before oocyte retrieval may affect the development of the embryo itself and thereby the implantation capacity of the embryo. Studies of possibly adverse effect of high E2 concentrations on developing preimplantation embryos are, however, inconclusive. Some studies were unable to find adverse effects (Papageorgiou et al., 2002; Kyrö et al., 2009; Imudia et al., 2014), while others found adverse effects (Shapiro et al., 2012; Roque et al., 2013).

High E2 and progesterone concentrations as a result of multiple corpora lutea may accelerate the maturation of the endometrium (Nikas et al., 1999; Liu et al., 2010; Zaptantis et al., 2013) and thereby cause asynchronous timing of the maturation of the endometrium with the hatching stage of the embryo that is a prerequisite for implantation. Both E2 and progesterone seem to influence the endometrial expression of leukemia inhibitory factor (LIF) and its receptor, which are vital for a successful implantation (Stewart et al., 1992; Cheng et al., 2002). In experimental models, E2 was found to have an inhibitory effect on LIF expression while progestins had a stimulatory effect (Bamberger et al., 1997a,b). Sub-optimal conditions for embryo implantation, due to high periovulatory and early luteal phase E2 and progesterone concentrations, may therefore result in slower trophoblast proliferation and lower hCG concentrations.

Conclusions
In our study of 1047 successful pregnancies after IVF we found that number of cells in the transferred embryo was positively associated with maternal hCG concentrations in very early pregnancy whereas a number of cells in the transferred embryo was positively associated with low hCG concentrations. Our findings may have implications for interpretation of early hCG concentrations after IVF.

Acknowledgements
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Authors’ roles
T.G.T. provided data for the study, aided in planning, wrote and edited the manuscript. A.E. conceived the study, made the statistical analyses, wrote and edited the manuscript.

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Conflict of interest
None declared.

References


