Cell-free fetal DNA levels in pregnancies conceived by IVF

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BACKGROUND: Increased second-trimester levels of maternal serum HCG in IVF conceptions lead to an increased false-positive rate in Down syndrome screening. Increased levels of cell-free fetal DNA (cffDNA) in maternal plasma have been correlated with increased HCG levels. Our aim was to determine whether cffDNA levels are elevated in IVF pregnancies compared with natural pregnancies. METHODS: Sixteen archived second-trimester serum samples from IVF pregnancies were matched with five control samples from naturally conceived pregnancies per case, all carrying a singleton male fetus. cffDNA concentrations were measured by real-time PCR amplification of a Y chromosome sequence and compared with four standard second trimester serum screening markers (α-fetoprotein, estriol, HCG and inhibin A). RESULTS: Mean cffDNA levels for cases and controls were 57.9 and 57.1 genome equivalents/ml, respectively (P = 0.95). Mean observed rank (from 1 to 6) of cffDNA was 3.625 in the IVF conceived group, compared with an expected value of 3.5 (P = 0.53). No significant correlations were observed between cffDNA and serum markers. CONCLUSIONS: IVF does not affect levels of cffDNA, which appears to be independent of traditional screening markers (e.g. HCG). Therefore, cffDNA can be used as an additional serum marker (e.g. Down syndrome screening) without adjustment for IVF pregnancies.

Key words: DNA/IVF/pregnancy/prenatal diagnosis/serum screening markers

Introduction

In 1997, Lo and colleagues reported that circulating cell-free fetal DNA (cffDNA) was detectable and measurable in the plasma and serum of pregnant women (Lo et al., 1997). The quantity of cffDNA found in maternal plasma is associated with both fetal and maternal factors such as fetal aneuploidy, placental abnormalities, preeclampsia, labour and termination (Bianchi, 2004). The tissue of origin of fetal DNA in maternal plasma is not known for certain. One theory is that cffDNA is continuously released from the placenta into the maternal circulation, similar to HCG. By correlating cffDNA levels with maternal serum screening markers, researchers have tried to identify the origin of cffDNA. Two recent studies have reached different conclusions. Ohashi et al. demonstrated a strong association between cffDNA levels and HCG levels, while Farina et al. showed the cffDNA levels were independent of second trimester screening markers (Ohashi et al., 2002; Farina et al., 2003).

Maternal serum screening is used for non-invasive risk assessment of Down syndrome, trisomy 18 and neural tube defects (Muller et al., 2003; Wald et al., 2003). The second trimester ‘quadruple’ screen includes HCG and inhibin A, which are of placental origin, estriol (uE3), which is of fetoplacental origin, and α-fetoprotein (AFP), which is at least in part of fetal origin. A risk assessment for Down syndrome can be calculated using measurements of these markers with comparison to population standards, but before the test is interpreted, raw values are adjusted for maternal age, race, smoking history, diabetes, multiple gestation and IVF conception (Wald et al., 1997). The effect of IVF conception on the risk assessment for Down syndrome is higher than expected false-positive rate, due primarily to an increase in HCG values. The etiology for this is unclear (Heinonen et al., 1996; Ribbert et al., 1996; Lam et al., 1999; Wald et al., 1999; Liao et al., 2001; Maymon and Shulman, 2002).

To date no studies have examined the effect of IVF conception on maternal serum cffDNA levels. We conducted a blinded case–control study to compare the concentrations of cffDNA as well as serum screening markers in archived maternal serum samples from women carrying male fetuses who conceived via IVF with women who conceived naturally. We hypothesized that cffDNA, if derived from the placenta, might be elevated by IVF, as is the case for HCG concentrations.

Methods

Approval for this study was obtained from the investigational review boards at Tufts University and Women and Infants Hospital.

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Archived serum specimens from 16 pregnant women confirmed to be carrying a singleton male fetus conceived by IVF were obtained from the Division of Prenatal and Special Testing at Women and Infants’ Hospital, Providence, RI, USA. Each case was paired with five controls (n = 80) from women carrying singleton male fetuses who conceived naturally. Samples were matched for gestational age, duration of specimen storage, maternal race, diabetes and smoking status. All IVF pregnancies, and therefore controls, were non-black, non-diabetic and non-smokers. An investigator not involved in the DNA quantitation coded each set of matched samples. For each set of matched samples, one to two specimens from women carrying female fetuses were included to serve as negative controls (n = 19).

Blood samples were initially obtained for maternal serum screening between 15 and 19 weeks of gestation by a standardized clinical protocol. The concentrations of the four serum markers (AFP, uE3, HCG and inhibin A) for the cases and controls were previously obtained as part of the routine serum screening program. Residual specimens from the initial serum collection were stored at −20°C for this study.

Archived samples were thawed at 37°C and centrifuged at 13 500 g for 10 min. Three hundred and fifty millilitres of serum were used for DNA extraction with the QIAamp Mini Blood Kit (Qiagen, Valencia, CA, USA) with the ‘blood and body fluids protocol’, as described by the manufacturer with minor modifications. The DNA was eluted into a final volume of 50 ml of EA buffer (Qiagen) and stored at 4°C pending analysis. Standard precautions to prevent DNA contamination were followed.

The concentrations of cfDNA in samples were determined by real-time quantitative PCR (qPCR) using a Perkin-Elmer Applied Biosystems 7700 Sequence Detector (Perkin-Elmer, Foster City, CA, USA). Samples were analysed with DYS-I and β-globin probes as described previously (Wataganara et al., 2003). DYS-I is a Y chromosome sequence used as a unique fetal marker, β-globin was used to measure the total maternal and fetal DNA that was present in the sample.

Each sample was run in triplicate for DYS-I and in duplicate for β-globin with 5 ml of the extracted DNA sample. An amplification plot of cycle number and DRn (fluorescent intensity over background) were generated from each reaction well. Reference amplification curves were generated concurrently with the use of a titrated, purified male DNA sample. A standard calibration curve of threshold cycles (cycle at which the amplification curve attains the threshold DRn value) against reference DNA concentrations was generated. The initial quantity of DNA in each unknown sample was calculated based on the calibration curve, the elution volume from the DNA extraction column and the starting volume of serum. Final quantities were expressed in genome equivalents per millilitre (GE/ml), with a conversion factor of 6.6 pg of DNA equal to 1 GE (Lo et al., 1998). For each sample, the three absolute values of DYS-I cfDNA were analysed for intrasample variation. Intrasample variation was noted if the lowest value divided by the highest value was <0.6 or the average of the three values divided by the highest value was <0.8. In cases of intrasample variation, samples underwent repeat qPCR amplification. The median of all values for each sample was used for further calculation.

Descriptive statistics, including medians and 25th and 75th percentile ranges, were generated for all studied variables. The maternal serum screening markers and cfDNA levels were logarithmically transformed prior to the analysis. Pearson linear regression was used to estimate the relationship between the log-transformed cfDNA, gestational age, maternal age and weight, method of conception and maternal serum screening markers. A matched rank sum analysis comparing cfDNA concentration of each IVF case with its five matched controls was calculated by a paired non-parametric analysis. Therefore, the rank of each IVF case can range from 1 to 6 for each group of six samples. No difference in cfDNA concentration of the IVF cases compared with controls would yield an average rank of 3.5. To detect the differences in cfDNA, HCG, uE3, AFP and inhibin A serum concentrations between IVF and naturally conceived pregnancies, the data were examined with a three-factor, mixed-model analysis of variance, adjusting for sample group, matched sample set and sample run. Owing to the previously reported physiological increase in circulating cfDNA levels with advancing gestational age, cfDNA levels were adjusted for gestational age (Lo et al., 1998). Statistical significance was assigned when the P-value was <0.05. All statistical analyses were performed using SAS/STAT software (SAS Institute, Inc., Cary, NC, USA).

Results

The mean cfDNA concentrations for controls and cases were 57.1 and 57.9 GE/ml, respectively (P = 0.95; Table I). Patients who conceived by IVF were older than those who conceived naturally (33.1 versus 28.7 years; P < 0.0001). There was no statistically significant difference in maternal weight or gestational age between cases and controls. Two of the samples from women carrying female fetuses had trace evidence of male DNA [one sample with a range of 0–3.12 pg/reaction well (median 1.10 pg/reaction) and the other sample with a range 0–1.25 pg/reaction (median 0 pg/reaction)]. In the 96 samples from women carrying male fetuses, only two did not have demonstrable amplification of the Y chromosome sequence. In this study, the sensitivity and specificity of gender detection was 97.9% and 89.5%, respectively. Owing to intrasample variation, 64 of 115 samples (55.7%) were re-analysed.

The median cfDNA concentrations increased as a function of gestational age (Figure 1). The Pearson correlation coefficient when comparing cfDNA levels and gestational age for the control pregnancies was 0.21. After including the IVF pregnancies, the correlation coefficient was 0.18. The difference between the controls and IVF cases was not significantly different (P = 0.57).

Table I. Patient demographics

<table>
<thead>
<tr>
<th></th>
<th>IVF pregnancies (average ± SD)</th>
<th>Controls (average ± SD)</th>
<th>P-value</th>
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</thead>
<tbody>
<tr>
<td>cfDNA (GE/ml)</td>
<td>57.9 ± 44.8</td>
<td>57.1 ± 47.6</td>
<td>0.95</td>
</tr>
<tr>
<td>Maternal age (years)</td>
<td>33.1 ± 3.1</td>
<td>28.7 ± 6.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>16.9 ± 0.8</td>
<td>17.0 ± 0.9</td>
<td>0.91</td>
</tr>
<tr>
<td>Maternal weight (pounds)</td>
<td>150.5 ± 42.2</td>
<td>147.5 ± 29.1</td>
<td>0.79</td>
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</tbody>
</table>
Quantitative DYS-1 amplification data were examined with a matched rank sum analysis (Figure 2). The mean observed rank of the median serum cffDNA concentrations from each of the IVF-conceived samples was 3.625, which was not significantly higher than the expected rank of 3.5 ($P = 0.53$). Using a mixed-model analysis of variance, there was no statistical difference in cffDNA concentrations between the IVF and naturally conceived groups ($P = 0.87$; Table II), although the median value was 10% higher in the IVF group. The median concentrations of AFP (1.00 versus 1.51 multiples of the median; MoM), HCG (0.95 versus 1.08 MoM), uE3 (0.97 versus 1.02 MoM) and inhibin A (0.77 versus 0.97 MoM) were less in IVF-conceived pregnancies than control pregnancies. Only inhibin A was found to have a borderline negative association with IVF conception ($P = 0.09$) while the other markers had $P$-values ranging from 0.25 to 0.90, and were therefore not significantly different between IVF-conceived pregnancies and control pregnancies. There were no significant correlations between cffDNA and each of the four maternal serum screening markers (HCG, AFP, uE3 and inhibin A) ($P = 0.18, 0.22, 0.57$ and 0.25, respectively). Because prior studies have shown increased HCG levels in IVF pregnancies, our samples were divided into two groups based on HCG levels; $< 1$ or $\geq 1$ MoM. A multivariate regression analysis, controlling for gestational age, showed that IVF conception and HCG values had no statistical impact on log-transformed cffDNA levels ($P = 0.43$).

**Discussion**

Our data indicate that IVF does not affect cffDNA levels in maternal serum, which disproved our original hypothesis. Nonetheless, the results of this study show that when compared with the traditional second trimester screening markers, cffDNA levels function as an independent serum marker. If cffDNA, in the future, is used as an additional serum marker (e.g. in Down syndrome screening), our data suggest that cffDNA values will not require adjustment for IVF conception. However, the current study is small and therefore has insufficient power to draw firm conclusions. Consequently, the results of this study suggesting that cffDNA is an independent screening marker require confirmation using a larger number of subjects.

Researchers have tried to identify the origin of cffDNA by studying the association between fetal DNA and maternal serum screening markers. Ohashi and colleagues reported that levels of cffDNA were strongly correlated with HCG levels (Ohashi et al., 2002). From 31 cases, the raw values for cffDNA and HCG levels had a Pearson correlation coefficient of 0.73 ($P < 0.0001$), suggesting that cffDNA may be of similar placental origin as HCG. Farina and colleagues reported no correlation between cffDNA and each of the four quadruple screening markers (AFP, uE3, HCG and inhibin A) studying serum samples from 16 pregnancies with fetuses with Down syndrome and 80 control pregnancies (Farina et al., 2003). Our study also did not find any significant correlation between cffDNA and serum markers, supporting the theory that cffDNA is an independent screening marker and may derive

![Figure 1](image1.png)

**Figure 1.** Median cffDNA concentrations from serum samples increase with gestational age. IVF pregnancies are represented by open circles and controls by filled circles. The continuous line and the dashed line represent the log-linear trend for control ($r = 0.21$) and all ($r = 0.18$) pregnancies, respectively.

![Figure 2](image2.png)

**Figure 2.** Results of rank sum analysis of the serum samples. The number of IVF cases with each numerical rank is represented by the bar graph. The mean observed rank was 3.625 and the mean expected rank was 3.5 ($P = 0.53$).

**Table II.** cffDNA and serum screening marker concentrations in IVF versus control pregnancies

<table>
<thead>
<tr>
<th></th>
<th>IVF pregnancies [median (25th, 75th percentile)]</th>
<th>Controls [median (25th, 75th percentile)]</th>
<th>Adjusted P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>cffDNA</td>
<td>48.87 (30.20, 73.12)</td>
<td>44.40 (20.48, 86.49)</td>
<td>0.87</td>
</tr>
<tr>
<td>AFP</td>
<td>32.50 (27.48, 41.70)</td>
<td>34.65 (28.98, 44.48)</td>
<td>0.90</td>
</tr>
<tr>
<td>uE3</td>
<td>0.77 (0.51, 0.91)</td>
<td>0.78 (0.66, 0.99)</td>
<td>0.25</td>
</tr>
<tr>
<td>HCG</td>
<td>26.01 (17.35, 31.56)</td>
<td>26.55 (19.41, 38.00)</td>
<td>0.50</td>
</tr>
<tr>
<td>Inhibin A</td>
<td>126.33 (105.68, 169.83)</td>
<td>178.87 (178.87, 231.94)</td>
<td>0.09</td>
</tr>
</tbody>
</table>

*Log-transformed data adjusted for gestational age, maternal weight and maternal age.
from more than one tissue source. This is further supported by recent evidence that placental mass does not correlate with cffDNA levels (Wataganara et al., 2005). However, a lack of correlation between cffDNA and serum markers does not necessarily exclude the same tissue source (e.g. cffDNA may correlate with number of cells while serum markers may correlate with level of expression), and additional investigation is needed to determine the tissue origin(s) of cffDNA.

Farina and colleagues also reported that with the addition of maternal serum cffDNA levels to second trimester serum screening, the detection rate of Down syndrome could improve from 81% to 86% at a fixed 5% false-positive rate (Farina et al., 2003). The median cffDNA MoM was 71% higher in pregnancies carrying fetuses with Down syndrome. Others have demonstrated 200–300% higher median fetal DNA concentration in pregnancies with fetuses with Down syndrome (Lo et al., 1999; Zhong et al., 2000). In our study, examining only unaffected pregnancies, the median cffDNA concentration was 10% higher in IVF conceived pregnancies, but the difference was not significant (P = 0.87). Therefore, it is unlikely that the use of cffDNA in screening of IVF pregnancies will have an impact on either the false-positive rate or the detection rate. To test this hypothesis, a future study would likely require multiple centres to accumulate a large enough population with several IVF pregnancies carrying a fetus with trisomy 21.

During this study, some potential confounders were encountered. At the time of second trimester serum screening, all cases and controls were identified as singleton pregnancies. The original number of transferred embryos, as well as spontaneous or elective reductions, was unknown. Multiple gestation pregnancies could theoretically increase concentrations of both cffDNA and serum screening markers, but this would have then affected values of both cases and controls (Smid et al., 2003). However, increasing HCG and cffDNA levels may apply more to IVF than spontaneous pregnancies, as multiple gestation pregnancies are more common after IVF than natural conception, and therefore vanishing twins may be more common following IVF. Additional investigation is necessary to fully assess these potential differences.

To date no studies have addressed the issue of intrasample variability in qPCR for cffDNA. This variability, as defined in the Methods section, was detected in 54% of initial qPCR runs. Variability between wells in a qPCR run for cffDNA is a concern recognized by most laboratories. Additionally, while DNA extraction and qPCR techniques are reliable and reproducible, they are dependent on a well-trained technician, as variability in quantitative and qualitative analysis between laboratories has been observed (Johnson et al., 2004). This concern was addressed in that study by repeating samples with intrasample variability and including these results in the final median calculation of GE/ml.

In this study, except for an indication that inhibin A was lower, the levels of the other three serum screening markers were essentially the same in both IVF and naturally conceived pregnancies. Several studies have reported an increase in HCG levels in IVF pregnancies, which may account for the increased false-positive detection rate of Down syndrome. AFP and uE3 levels have a wide range of reported values (0.88–1.13 MoM and 0.90–1.11 MoM, respectively) (Barkai et al., 1996; Heinonen et al., 1996; Ribbert et al., 1996; Frishman et al., 1997; Lam et al., 1999; Wald et al., 1999; Maymon and Shulman, 2002; Muller et al., 2003). These variable results are likely due to small sample populations (n = 41–69), because in two studies with larger sample populations (n = 327 and 1515) the median values of AFP and HCG were not statistically different from controls (Barkai et al., 1996; Muller et al., 2003). One study found slightly decreased values of HCG, AFP and uE3 in IVF pregnancies (Barkai et al., 1996).

Based on our results, cffDNA levels in maternal serum do not appear to be affected by IVF conception, and therefore may not need adjustment for pregnancies achieved by IVF compared with naturally conceived pregnancies. In addition, since cffDNA appears to be an independent maternal serum marker compared with the traditional second trimester ‘quadruple’ markers (HCG, AFP, inhibin A and uE3), our data also suggest that cffDNA may have a different tissue source from these markers or may have more than one tissue origin.

Acknowledgements

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References


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