Maternal serum inhibin A concentrations in early pregnancy after IVF and embryo transfer reflect the corpus luteum contribution and pregnancy outcome

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To compare maternal serum inhibin A concentrations in early pregnancy with pregnancy outcomes and treatment protocols, serum samples were collected from 237 women undergoing in-vitro fertilization (IVF) and embryo transfer cycles. Samples were collected on day 16 after oocyte retrieval for β human chorionic gonadotrophin (HCG) pregnancy testing and inhibin A measurement. The samples were divided into non-pregnant (n = 128) and pregnant (n = 109) groups, the pregnancies were followed and outcomes determined. Inhibin A concentrations were significantly lower in non-pregnant women than in women with ongoing pregnancies (P < 0.001) and those resulting in spontaneous abortions (P < 0.001). In ongoing pregnancies, inhibin A concentrations were significantly lower in the absence of functioning ovaries (donor oocyte/embryo) (P < 0.01) and in natural cycles (frozen–thawed embryo transfer) (P < 0.01) compared with concentrations after ovarian stimulation. Further, since inhibin A concentrations were not significantly different between singleton and multiple pregnancies in the ovarian stimulation protocol, the size of the early trophoblast does not appear to influence the secretion of inhibin A. These data strongly support the concept that the corpus luteum is a major source of circulating inhibin A in early pregnancy. Additionally, low concentrations of serum inhibin A may be useful in predicting βHCG-positive preclinical ‘biochemical’ pregnancies.

Key words: corpus luteum/inhibin A/in-vitro fertilization/outcomes/pregnancy

Introduction
Inhibins are heterodimeric proteins composed of an α and one of two β subunits, βA or βB, giving rise to two isoforms inhibin A (αβA) and inhibin B (αβB) and are defined by their ability to inhibit FSH secretion from the pituitary (de Kretser and Robertson, 1989). During the human menstrual cycle, immunoreactive (ir) inhibin was shown to be produced by the dominant follicle and the corpus luteum peaking in the luteal phase (McLachlan et al., 1987; Lenton et al., 1991) and, subsequently, this was shown to be inhibin A (Groome et al., 1994; Muttukrishna et al., 1994). Inhibin B is secreted from the developing follicles during the menstrual cycle, peaking in the follicular phase (Groome et al., 1996; Magoffin and Jacimuk, 1997). During human pregnancy, circulating concentrations of inhibin B are low to undetectable (Illingworth et al., 1996; Fowler et al., 1998), whereas ir-inhibin and inhibin A have been shown to rise across gestation particularly in the third trimester (Yohkaichiya et al., 1991; Muttukrishna et al., 1995) reflecting a feto-placental origin in later pregnancy. However, there are conflicting reports regarding the source of inhibin A in the initial stages of pregnancy.

During the establishment of pregnancy, the corpus luteum plays a major role in maintenance of early gestational milieu but its role gradually is replaced by that of the trophoblast and developing placenta. During early pregnancy, mRNAs for α, βA and βB subunits have been demonstrated in the follicles, corpus luteum (Roberts et al., 1993) and the trophoblast (Meunier et al., 1988; Baird and Smith, 1993). Immunohistochemical studies have demonstrated the presence of α, βA and βB subunits in the corpus luteum and placental tissues (Petraglia et al., 1991; Yamoto et al., 1991; Minami et al., 1992; McCluggage et al., 1998). Previous studies have reported a major contribution of circulating inhibin from either the ovary (Yohkaichiya et al., 1991; Illingworth et al., 1996; Rombouts et al., 1996) or the placenta (Birdsall et al., 1997; Lockwood et al., 1997; McLachlan et al., 1987; Muttukrishna et al., 1997) in spontaneous and in-vitro fertilization (IVF)–embryo transfer pregnancies. The apparent discrepancies appear to be due, in part, to the different assays used, the stage of gestation and possibly the different hormonal treatment protocols used in the IVF–embryo transfer cycles of women enrolled in these studies. The early studies used a radioimmunoassay which, in addition to measuring the dimeric inhibins, measured the monomeric free α subunit (Robertson et al., 1989). Recently, a sensitive and specific two-site enzyme linked immunosorbent assay for inhibin A has been developed (Groome et al., 1994) which has enabled the study of this protein specifically.

Different patterns of circulating inhibin A (Lockwood et al., 1997) and pro-αC (the pro region of the α subunit) (Illingworth et al., 1996) have been shown to be potential markers of pregnancy outcomes indicating the presence of non-viable pregnancies. Several studies have attempted to relate parameters including inhibin A to ovarian reserve and subsequent IVF pregnancy outcomes (Barnhart and Osheroff, 1998; Broekmans et al., 1998; Hall et al., 1999). However, the
dynamics of inhibin A secretion during establishment of pregnancy may be more useful in predicting IVF pregnancy outcomes.

Therefore, this study, in a large number of patients, was undertaken to investigate whether serum inhibin A concentrations on day 16 after oocyte retrieval in IVF-embryo transfer cycles are correlated with subsequent pregnancy outcomes, and to determine whether different treatment protocols which reflect different levels of corpus luteum function affect the circulating concentrations of inhibin A in early pregnancy.

Materials and methods

Subjects and samples

The study was performed retrospectively on blood samples routinely collected on day 16 after oocyte retrieval for the diagnosis of pregnancy following IVF and embryo transfer. Permission for the use of these samples for this study was given by the Ethics Committee of the Epworth Hospital (Richmond, Victoria, Australia). After the aliquots were used to measure human chorionic gonadotrophin (HCG) concentrations for pregnancy tests, the samples were stored at −20°C for further assays.

Treatment protocols

A variety of hormonal treatment protocols was used in IVF–embryo transfer cycles.

Frozen–thawed cycles

(i) In women without functioning ovaries, embryo transfer was achieved using donated oocytes or frozen–thawed embryos. The endometrium was prepared using a hormonal replacement (HRT) protocol whereby 4 mg daily orally of oestradiol valerate (Progynova®; Schering, Sussex, UK) was prescribed for 12–20 days. After embryo transfer, additional luteal support was provided by 400–600 mg daily progesterone vaginal suppositories at least until serum samples were collected. One woman, with some ovarian function but with repeated IVF failures, underwent pituitary desensitization using a GnRH agonist, nafarelin (Synarel®; Searle, High Wycombe, UK) prior to preparation of the endometrium using the HRT protocol as above, and embryo transfer using a donated frozen–thawed embryo. (ii) In patients with functioning ovaries and regular menstrual cycles, replacement of their frozen embryos occurred using a natural cycle (NAT) in which the endometrium was prepared by endogenous oestradiol and progesterone.

IVF–embryo transfer cycles

Two treatment protocols were used: (i) ovarian minimal stimulation using clomiphene citrate; or (ii) ovarian stimulation, in which nafarelin was prescribed for pituitary desensitization and was followed by injection of human recombinant follicle stimulating hormone (rhFSH) (Gonal-F®; Ares-Serono, Geneva, Switzerland). When the dominant follicle or follicles were >17 mm diameter, nafarelin and rhFSH were discontinued and prior to oocyte retrieval, 5000–10 000 IU s.c. of HCG (Profasi®; Serono Laboratories, Geneva, Switzerland) was administered for oocyte maturation to subjects with clomiphene or ovarian stimulation protocols. After embryo transfer, luteal support with 400–600 mg daily of progesterone vaginal suppositories was prescribed for all women and continued at least until the samples were collected.

Pregnancy outcomes

Pregnancy was diagnosed on day 16 after oocyte retrieval by the detection of 10 mIU/ml or higher of serum βHCG concentrations and the samples were divided into two groups: non-pregnant (n = 128) and pregnant (n = 109). All pregnancies were followed for a minimum of 12 weeks of gestation and were subsequently classified into four groups: (i) preclinical (Pcln, n = 15); (ii) ectopic (Ect, n = 2); (iii) spontaneous abortion (Sp. Ab, n = 12); and (iv) ongoing (n = 80) pregnancies. A preclinical ‘biochemical’ pregnancy was diagnosed if weekly βHCG concentrations declined and no fetal heart beat (FHB) was detected at 6–7 weeks of gestation by transvaginal ultrasonography. Diagnosis of an ectopic pregnancy was made if FHB was detected outside the uterine cavity. Spontaneous abortion was diagnosed if the previously positive intrauterine FHB became undetectable or pregnancy spontaneously terminated after 6–7 weeks of gestation. Ongoing pregnancy was diagnosed if the pregnancy continued beyond 12 weeks of gestation.

Further, all ongoing pregnancies were divided into four groups based on the hormonal treatment protocols as described above: (i) natural cycle (NAT, n = 12); (ii) hormone replacement (HRT, n = 4); (iii) clomiphene citrate (clomiphene, n = 4); and (iv) ovarian stimulation. The ovarian stimulation group (n = 60) was further subdivided into groups with viable singleton (VS, n = 39) or viable multiple fetus (VM, n = 21) pregnancies.

Assays

βHCG was measured using a commercial assay kit (Vitros free βHCG; Ortho-Clinical Diagnostics, Amersham, UK). The sensitivity of the assay was 0.04 mIU/ml. Inhibin A was measured by specific methods previously described elsewhere (Groome et al., 1994) using WHO inhibin standard 91/624. The sensitivity of the assay was 4 pg/ml. Intra- and inter-plate assay coefficients of variation were 9.2 and 10.6% respectively.

Statistical analysis

The data are presented as arithmetic mean ± SEM and analysed using Kruskal–Wallis non-parametric analysis with Dunn’s post hoc test. Inhibin A measurements lower than the detection limit of 4 pg/ml were assumed to be 4 pg/ml for statistical purposes. Analyses were carried out using the GraphPad Prism 2.01 software package (GraphPad software Inc., San Diego, CA, USA). Ratios were calculated with matched data for each patient and the mean of these is shown.

Results

Serum inhibin A and βHCG concentrations in women who were subsequently diagnosed with ongoing pregnancies were significantly higher than in non-pregnant women (P < 0.001) (Figure 1). Women with pregnancies later diagnosed as preclinical had significantly lower inhibin A concentrations (P < 0.01) than those with ongoing pregnancies but no significant difference in βHCG concentrations was noted. Inhibin A concentrations in women with pregnancies resulting in spontaneous abortions were not significantly different to the concentrations in ongoing pregnancies. However, in these cases, a lower inhibin A to βHCG ratio (1.08 ± 0.41) than that seen in ongoing pregnancies (1.61 ± 0.26) was noted, but this difference was not significant. No significant differences in inhibin A concentrations were found between the group of women with ectopic and those with ongoing pregnancies. However, a high inhibin A to βHCG ratio was noted in the small number of women with ectopic pregnancies (8.74 ± 1.53), similar to that found in non-pregnant women (9.58 ± 0.67).

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Circulating serum inhibin A and βHCG concentrations on day 16 after oocyte retrieval in women with pregnancies subsequently diagnosed as ongoing, after various treatment protocols: endometrium prepared by hormonal replacement (HRT) or natural cycle (NAT), ovarian minimal stimulation by clomiphene citrate (CC) and ovarian stimulation (COH) resulting in viable singleton (VS) and viable multiple (VM) fetus pregnancies. Data are shown as arithmetic mean ± SEM. Different letters denote statistical significance ($P < 0.05$) using Kruskal–Wallis non-parametric analysis with Dunn’s post hoc test.

Figure 2. Circulating serum inhibin A and βHCG concentrations on day 16 after oocyte retrieval in women with pregnancies subsequently diagnosed as ongoing, after various treatment protocols: endometrium prepared by hormonal replacement (HRT) or natural cycle (NAT), ovarian minimal stimulation by clomiphene citrate (CC) and ovarian stimulation (COH) resulting in viable singleton (VS) and viable multiple (VM) fetus pregnancies. Data are shown as arithmetic mean ± SEM. Different letters denote statistical significance ($P < 0.05$) using Kruskal–Wallis non-parametric analysis with Dunn’s post hoc test.

Figure 1. Circulating serum inhibin A (a) and βHCG (b) concentrations, and ratio of inhibin A:βHCG (c) in non-pregnant women (NP) and women subsequently classified with preclinical (Pcln), ectopic (Ect) pregnancies, spontaneous abortions (Sp.Ab) and ongoing (Ongoing) pregnancies. Data are shown as arithmetic mean ± SEM. Ratios (c) were calculated from matched data from each patient. Different letters denote statistical significance ($P < 0.05$) using Kruskal–Wallis non-parametric analysis with Dunn’s post hoc test.

Analysis of serum inhibin A and βHCG concentrations in women with ongoing pregnancies, according to the type of stimulation used in their IVF/embryo transfer cycle showed significant differences in circulating inhibin A concentrations between some groups (Figure 2). However, there was no significant change in the βHCG concentrations between treatment groups. Inhibin A concentrations were significantly lower in cases where the women who received the HRT protocol had no corpus luteum ($P < 0.01$) and during natural cycles (one corpus luteum) ($P < 0.01$) compared with those women receiving the ovarian stimulation protocol (multiple corpora lutea). Inhibin A concentrations in women who received the ovarian stimulation protocol resulting in viable singleton and viable multiple pregnancies were not significantly different. In contrast, βHCG concentrations were significantly higher in women with viable multiple pregnancies ($P < 0.05$) in comparison with those with viable singleton pregnancies (Figure 2).

Discussion

This study demonstrates that serum inhibin A concentrations in early pregnancy (day 16 after oocyte retrieval and the day of the pregnancy test in IVF–embryo transfer programmes) reflect ovarian function and provide evidence that inhibin A is a product not only of the feto-placental unit but also of the corpus luteum in early pregnancy. Further, the circulating concentrations reflect some pregnancy outcomes.

In early pregnancy, it has been shown that $\alpha$ and $\beta_A$ subunits are expressed in both the corpus luteum and trophoblast (Yamoto et al., 1991; Minami et al., 1992). To investigate the source of circulating inhibin A in the early stages of gestation, a variety of hormonal treatment protocols used in IVF–embryo transfer programmes and the number of fetuses in successful pregnancies were examined. The different treatment protocols reflect different numbers of functional corpora lutea, whereas the number of viable fetuses reflects the mass of the trophoblast.

Inhibin A concentrations in pregnant women reflected the number of corpora lutea arising from different IVF–embryo transfer treatments. The inhibin A concentrations were significantly lower in cases where pregnancies occurred in women without functioning ovaries (HRT protocol) reflecting the absence of a functional corpus luteum, and in women where the endometrium was prepared by NAT protocol (single corpus luteum), compared with those women who received the ovarian stimulation protocol (multiple corpora lutea). Further, there was no significant difference in inhibin A concentrations between viable singleton and multiple pregnancies. However, the increased trophoblast mass in multiple pregnancies was reflected in significantly higher βHCG concentrations compared with singleton pregnancies. It is possible that the increased output of inhibin A from multiple corpora lutea may
be obscuring the differences in inhibin A secreted from the trophoblast at this stage of pregnancy. These findings support prior studies (Santoro et al., 1992; Yohkaichiya et al., 1993; Rombauts et al., 1996) which showed that the corpus luteum was a source of circulating inhibins very early in pregnancy.

It has been shown that inhibin production is increased in the corpus luteum stimulated by exogenous HCG (Illingworth et al., 1990). Illingworth et al. (1996) have demonstrated that the luteal phase corpus luteum is capable of increased and continued secretion of inhibin A but not inhibin B when stimulated by exogenous HCG in non-pregnant women, indicating the ovary is a potential source of inhibin A in early pregnancy. Rombauts et al. (1996) have shown higher inhibin A concentrations in early pregnancies after IVF–embryo transfer than seen in spontaneous conceptions. The women in this study received HCG for luteal support which, despite comparable circulating concentrations of HCG in spontaneous and IVF pregnancies, may have influenced the inhibin A concentrations in the IVF pregnancies. However, our study has shown a similar observation of higher inhibin concentrations when comparing pregnancies resulting from natural cycles compared to ovarian stimulation, with both groups receiving only progesterone for luteal support. To our knowledge, luteal support using progesterone has an effect directly by supporting the secretory endometrium but no significant effect on function of the corpus luteum or trophoblast has been demonstrated.

Other studies have concluded that the placenta is the source of circulating concentrations on inhibin A in early pregnancy. Muttukrishna et al. (1997) showed a decrease in inhibin A concentrations after termination of pregnancy. They concluded that the removal of the feto-placental unit also removed the source of inhibin A. However, the removal of the fetus and placenta may also have resulted in the removal of one or more sources of stimulation to the ovary. Both inhibin A and progesterone fell to luteal values after the procedure, indicating the corpus luteum was still functional at this early stage of gestation. Recent studies have demonstrated similar inhibin A concentrations in pregnancies resulting from donor eggs and spontaneous conceptions (Birdsall et al., 1997) and fresh IVF–embryo transfer compared to spontaneous conceptions (Lockwood et al., 1997).

It is clear that in the latter stages of pregnancy, in particular the third trimester, the feto-placental unit is the major source of inhibin A with a significant rise to term (Muttukrishna et al., 1995), concomitant with the rapidly expanding feto-placental mass.

The observations from the present study on a large group of women clearly demonstrate that the corpus luteum is a major source of inhibin A in early pregnancy. These data, using an assay specific for inhibin A, expand and confirm an earlier study (Yohkaichiya et al., 1993) which used an assay that in addition to measuring dimeric inhibin, cross-reacted with the free α subunit products. The data also indicate that the measurement of inhibin A can provide an index of corpus luteum function in women receiving progesterone concomitantly.

The concentrations of circulating inhibin A were higher in pregnancies likely to be ongoing whereas significantly lower concentrations were found in non-pregnant subjects and pregnancies later classified as preclinical ‘biochemical’ pregnancies. Although not significantly different, inhibin A concentrations were lower in pregnancies resulting in spontaneous abortions compared to those in ongoing pregnancies. These results are similar to a prior study with smaller numbers of women (Lockwood et al., 1997) which showed that low circulating inhibin A concentrations at the time of the first pregnancy test (4 weeks of gestation) were related to poor IVF pregnancy outcomes. In our study, however, inhibin A concentrations within the groups were quite variable and showed overlap between groups. Therefore, these concentrations cannot be used at this point directly to predict pregnancy outcomes. A larger number of cases is needed to define the ranges of inhibin A concentrations at specific times after embryo transfer and after differing ovarian stimulation regimes before diagnostic criteria are developed. Given the enormous variety of stimulation protocols and varying ovarian sensitivities, all of which may modify the mass of luteal tissue in the ovary after oocyte retrieval, the definition of such ranges may prove difficult.

Inhibin A concentrations in a small number of cases consequently diagnosed as ectopic pregnancies were not significantly different from those in cases later diagnosed as ongoing pregnancies, but a high inhibin A to βHCG ratio was noted, similar to that seen in non-pregnant women. However, a larger number of cases is needed to demonstrate this significance in ectopic pregnancies.

In conclusion, these findings demonstrate that, in addition to the trophoblast, the ovary is also a significant source of circulating inhibin A in early pregnancy. The study also shows that, whilst inhibin A concentrations at the time of pregnancy test, 16 days following oocyte retrieval in IVF–embryo transfer cycle, may not be useful for predicting poor pregnancy outcomes such as ectopic pregnancies or spontaneous abortions, inhibin A may, however, be a useful marker in predicting βHCG positive ‘biochemical’ preclinical pregnancies.

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