Maternal Serum Steroid Levels Are Unrelated to Fetal Sex: A Study in Twin Pregnancies

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Increased prenatal exposure to testosterone (T) in females of an opposite-sex (OS) twin pair may have an effect on the development of sex-typical cognitive and behavioral patterns. The prenatal exposure to T due to hormone transfer in OS twin females may occur in two ways, one directly via the feto–fetal transfer route within the uterus, the other indirectly through maternal–fetal transfer and based in the maternal–fetal compartment. Although some studies in singletons indeed found that women pregnant with a male fetus have higher T levels during gestation than women pregnant with a female fetus, many other studies could not find any relation between the sex of the fetus and maternal serum steroid levels. Therefore at present it is unclear whether a pregnant woman bearing a male has higher levels of T than a woman bearing a female. Up to this point, no-one has investigated this issue in twin pregnancies. We examined the relationship between maternal serum steroid levels and sex of fetus in 17 female–female, 9 male–male and 29 OS twin pregnancies. No differences were observed between the maternal serum steroid levels of women expecting single-sex and mixed-sex offspring. It is concluded that the source of prenatal T exposure in females probably comes from the fetal unit, which is the direct route of fetal hormone transfer.

Research on normal healthy twins may provide an opportunity to investigate the possible prenatal effects of testosterone (T) on brain and behavior. In animal research it has been shown that exposure to steroids is influenced by the intrauterine position of the fetus. Female fetuses located between two male fetuses are exposed to higher levels of T than fetuses situated between two females or one female and one male fetus (Gandelman, 1992; Vom Saal, 1989; for review, see Ryan & Vandenbergh, 2002). Fetal hormone transfer may occur in two ways: (1) more indirectly via the maternal–fetal transfer route, as Meulenberg and Hofman (1991) described; and (2) more directly via the feto–fetal transfer route. With respect to the latter route, it has been shown in rodents that T can diffuse across amniotic membranes (e.g., Even et al., 1992). At present it is unclear whether similar processes operate in human opposite-sex (OS) fetal twins. Evidence in support of an increased prenatal T exposure effect in female animals that were located between male fetuses comes from the observation that these female animals have anatomical characteristics and show behavioral patterns more typical of males (Ryan & Vandenbergh, 2002). The few studies that have been conducted in human opposite-sex twins suggest that female fetuses of an OS twin-pair could have been masculinized in a similar way (see Miller, 1998, for review). For example, it has been found that OS twin females have a number of spontaneous otoacoustic ear emissions (McFadden, 1993) and tooth sizes (Dempsey et al., 1999) that are more similar to males than to females. Also, OS twin females seem to have more masculine attitudes compared to same-sex twin females with regard to sensation-seeking behavior (Resnick et al., 1993).

Although it is presently unclear how the increased prenatal T exposure — if it indeed exists — is caused, it presumably occurs through direct or indirect hormone transference between the OS fetuses.

In the 1970s and early 1980s of the last century several investigators tried to establish a link between fetal sex and maternal T levels (Bammann et al., 1980; Dawood & Saxena, 1977; Forest et al., 1971; Glass & Klein, 1981; Klinga et al., 1978; Nagamani et al., 1979; Rivarola et al., 1968; Rodeck et al., 1985). At the time, this information was considered

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to be useful in the antenatal determination of fetal sex, which in turn would be a fairly inexpensive screening tool in, for example, the diagnosis of sex-linked genetic disorders (Glass & Klein, 1981). However, this did not seem to be very productive because of inconclusive results and an extensive overlap between the sexes.

Years later, Meulenberg and Hofman (1991) investigated the relationship between fetal sex and maternal T serum concentrations again and concluded that male fetus pregnancies were associated with higher maternal serum T levels in the second half of the pregnancy. The maternal serum T levels differed at least 1 standard deviation in this period among male/female fetus pregnancies. Like Bammann et al. (1980), they claimed that this could be due to 'a gradient from the fetal to the maternal compartment at the level of plasma unbound testosterone' (Meulenberg & Hofman, 1991, p. 53). So far, all the previously conducted studies have focused on singleton pregnancies. Since the maternal–fetal gradient of unbound T is able to cross the placenta from the male fetus towards the maternal blood circulation it would be of interest to investigate whether maternal serum T levels reflect the number of male fetuses the pregnant mother is carrying in her womb. If this is the case, then women carrying male–male twins should have higher T levels than women carrying OS twins, and the latter group should again have higher levels than women expecting female–female twins.

In that case, this may reflect the assumption of the (indirect) maternal–fetal route of hormone transfer between co-twins. This could be the hormonal basis for the unusual hormonal milieu wherein OS twin females are found, and may lead to the rather atypical sex patterns that are found in females with a co-twin brother.

In summary, this present study focused on whether the sex of the fetus is associated with maternal serum steroid levels in twin pregnancies. This association may be an indication of the possible indirect route, that is via the maternal–fetal compartment, of T exposure through hormone transfer during twin pregnancies.

Research in the past has shown that subfertile women may have different endocrine profiles to women without fertility problems. For example, in women with polycystic ovary syndrome, a common disorder in subfertile women, one of the three possible criteria for diagnosis are the biochemical features of hyperandrogenism (Agrawal et al., 2004). As some of the women that participated in the study became pregnant as a result of treatment for subfertility (nonspontaneous twin pregnancies), this is considered in the analysis in order to control possible effects of different endocrine profiles.

Materials and Methods

Subjects

All participants were recruited between August 2000 and August 2002 from the Department of Obstetrics at the University Medical Center in Utrecht (the Netherlands) after an ultrasonic examination had confirmed a twin pregnancy. The women were invited to participate in this study by information letter. From the 89 women who were invited to take part, 59 agreed to participate. The data of 55 women were finally used in this study as one woman had a miscarriage, one of the twins died during pregnancy for two women, and one woman withdrew from the study without giving a reason. Of these 55 women, 9 carried male–male (MM) twin pairs, 29 OS twin pairs and 17 female–female (FF) twin pairs. The average age was 33.9 years (*SD* = 3.4; range 25 to 41 years). Thirty-eight women were nulliparous. Some of the twin pregnancies were spontaneously conceived while others were induced (e.g., in-vitro fertilization; see Table 1). A written informed consent form was obtained from all participating women. The Medical Ethical Committee of the University Medical Center in Utrecht approved the study.

Table 1

The Numbers of Spontaneous and Nonspontaneous Pregnancies per Typed Twin

	FF	DOS	MM
Spontaneous	12	12	5
Nonspontaneous	4	17	4

Note: One conception form was not able to be retrieved.

Materials

Two samples of blood serum were obtained from the participants: one sample was taken at 24 weeks and one at 32 weeks of gestation. The sera were taken during a normal visiting appointment with the gynecologist. As a result of logistic reasons and the longitudinal character of this study, some data are missing. Table 2 presents an overview of the hormone samples. All samples were collected and stored at –30°C until assayed.

Several steroids were measured: Testosterone (T), Progesterone (Prog), Dehydroepiandrosterone-sulfate (DHEAS), Sex hormone–binding globulin (SHBG), Estradiol (E2), and Androstenedione (A). Free Testosterone (FT) was calculated.

Testosterone was measured after diethylether extraction using an in-house competitive radioimmunoassay (RIA) employing a polyclonal antitestosterone-antibody (Dr Pratt AZG 3290). [1, 2–^{3}H(N)]-Testosterone (NET-387, DuPont NEN Nederland BV) was used as a tracer following chromatographic verification of its purity. The lower limit of detection was 0.12 nmol/L and interassay variation was 7.6, 5.0 and 5.9% at 1.5, 4.9 and 24 nmol/L respectively (n = 45).

Androstenedione was measured after hexane-toluene extraction using an in-house competitive RIA employing a polyclonal antiandrostenedione-antibody (Dr Pratt AZG 271178). [1,2,6,7-^{3}H]-Androst-4-ene-3, 17ß-dione (TRK 454, Amersham Pharmacia

biotech) was used as a tracer following chromatographic verification of its purity. The lower limit of detection was 0.2 nmol/L and interassay variation was 9.0; 5.5 and 5.7% at 1.4; 5.0 and 11.5 nmol/L respectively (n = 20).

DHEAS was measured using an immunometric technique on an Advantage Chemiluminescence System (Nichols Institute Diagnostics, San Juan Capistrano, USA). The lower limit of detection was 0.1 µmol/L and interassay variation was 7.0; 5.9 and 4.9% at 1.0; 4.7 and 14.3 µmol/L respectively (n = 50).

SHBG was measured using an immunometric technique on an IMMULITE Analyzer (Diagnostic Products Corporation, Los Angeles, USA). The lower limit of detection was 5 nmol/L and interassay variation was 5.5; 4.1 and 5.3% at 14; 34 and 91 nmol/L respectively (n = 23).

E2 was determined using the Axsym of Abbott (Abbott Park, Ill. 60064). Sera was diluted with phosphate buffered saline (0.01 Mol at pH 7.0, containing 0.5% Bovine Serum Albumin; second trimester serum 20x, third trimester serum 40x). Interassay variation was 5.1% at 1060 pmol/L.

Prog was determined by the above-mentioned method with a dilution of 10x. Interassay variation was 5.0% at 18 nmol/L.

FT was calculated using the equations described by Dunn et al. (1981). These equations can be used to describe the relation between the bound and free fraction of T with other steroids and with several binding proteins. Calculations were performed for a system of two steroids (E2 and T) and two binding proteins (albumin and SHBG). The value of albumin in maternal serum was fixed at 35 g/L for measurements at 24 weeks gestation and 33 g/L at 32 weeks gestation.

Statistical Analyses

To establish that the maternal blood serum steroid levels indeed showed sex differences, the mean levels of the three possible groups were compared (female–female [FF], opposite-sex [OS], and male–male [MM]) at both time points (i.e., 24 and 32 weeks) using a repeated measures model. Separate analyses of repeated measures for the 'male' steroids (i.e., T, FT, A, DHEAS, SHBG) and for the 'female' sex hormones (i.e., E2 and Prog) were conducted.

Results

Sex

The pregnant women who participated in the study gave birth to 9 MM, 29 OS, and 17 FF twin pairs, that is, 47 male and 63 female which gives a sex ratio of .75. This is different from the sex ratio normally found in singletons, namely 1.045.

Group and Time Differences in Maternal Serum Steroids: T, FT, A, DHEAS, and SHBG

None of the levels of prenatal steroids displayed any statistically significant difference between the three groups (i.e., MM, OS and FF), and only a main time effect was found for FT (F[1, 31] = 5.29, p < .05), SHBG (F[1, 31] = 10.17, p < .01), and DHEAS (F[1, 31] = 5.30, p < .05). These data reflect the fact that in all groups there was a progressive increase in FT and SHBG during pregnancy, but a decrease in DHEAS (see Table 2). There were no statistically significant interactions between group and time, and it can therefore be concluded that the groups responded in the same manner to the effect of time for all hormones assessed.

Group and Time Differences in Maternal Serum Sex Hormones: E2 and Prog

There were no differences between the three twin groups in maternal serum levels of E2 and Prog. However, these hormone levels increased significantly over time: E2 (F[1, 34] = 39.85, p < .001) and Prog (F[1, 34] = 149.80, p < .001); see Table 2.

Former Analysis Including the Conception Form as a Covariate

The chi-square test was not significant for the distribution of type of twin between the different conception forms, that is, spontaneous and nonspontaneous. However, because of the possible interference of an abnormal endocrine profile in the nonspontaneous women, an extra analysis was entered in the model in the way of conception. This showed that only the SHBG (F[1, 29] = 5.91, p < .05) and Prog (F[1,32] = 15.02, p < .001) still increased significantly over time for all twin groups.

Discussion

According to the hypothesis of hormone transfer between co-twins (Miller, 1998), exposure to T *in utero* may occur via the maternal–fetal route apart from the feto–fetal route. This study was designed to find (indirect) support for this possibility. It was predicted that if the maternal serum steroid levels depend (at least partly) on the sexes of the twins in the womb, the mothers of MM twins would show different levels of steroids in their serum, as an indication of the hormone transfer via the maternal–fetal blood circulation, to mothers of OS twins or FF twins. In singleton pregnancies, differences in maternal serum levels of androgens have been found in women pregnant with males and women pregnant with females (e.g., Meulenberg & Hofman, 1991). There are also studies, however, which have failed to find such a relationship between fetal sex and maternal serum steroid levels (e.g., Nagamani et al., 1979; Rodeck et al., 1985).

In the present study we collected serum twice (i.e., in week 24 and 32 of gestation) in 9 women pregnant with MM twins, 29 women pregnant with OS twins, and 17 women pregnant with FF twins. We were unable to establish any difference in maternal serum steroid levels between mothers carrying a MM twin pair, FF twin pair or OS twin pair.

The present outcome together with previous nonfindings questions the reliability of the two studies in singletons that observed a difference in maternal

Table 2

Mean Maternal Steroid Sera Levels in Women Pregnant With Twins According to the Sex of the Newborn Twins

Steroid		Maternal serum at 24 weeks (*SD* in parentheses).	$N_{24weeks}$	Maternal serum at 32 weeks (*SD* in parentheses).	$N_{32weeks}$
DHEAS (μmol/L)	FF twin	3.07 (1.60)	15	2.45 (1.30)	12
	OS twin	2.99 (1.81)	27	2.09 (1.28)	22
	MM twin	3.04 (1.35)	7	2.74 (0.83)	7
Androstenedione (nmol/L)	FF twin	8.01 (4.31)	15	6.91 (4.82)	13
	OS twin	6.20 (2.79)	27	4.99 (2.60)	21
	MM twin	6.20 (2.82)	7	7.78 (5.41)	6
SHBG (nmol/L)	FF twin	403.00 (107.50)	15	455.83 (153.52)	12
	OS twin	434.44 (75.34)	27	465.23 (81.95)	22
	MM twin	452.14 (94.95)	7	496.43 (97.03)	7
Testosterone (nmol/L)	FF twin	5.26 (3.55)	15	3.79 (2.20)	12
	OS twin	3.95 (2.21)	27	2.96 (1.82)	22
	MM twin	3.50 (1.59)	7	5.06 (3.84)	7
Progesterone (nmol/L)	FF twin	482.67 (180.32)	15	894.83 (216.60)	12
	OS twin	493.00 (138.27)	27	1046.18 (301.84)	22
	MM twin	455.86 (163.76)	7	1104.29 (297.34)	7
Estradiol (pmol/L)	FF twin	117493 (37855)	15	182200 (66079)	12
	OS twin	115559 (50799)	27	152181 (55978)	22
	MM twin	106971 (24802)	7	182771 (57781)	7
Free Testosterone (pmol/L)	FF twin	70.68 (47.36)	15	77.56 (70.14)	11
	OS twin	48.58 (29.50)	27	55.23 (38.01)	22
	MM twin	41.00 (23.68)	7	93.46 (59.16)	7

Note: MM = male–male twin; OS = opposite-sex twin; FF = female–female twin.

serum steroid levels, although both did not agree on the period of the pregnancy in which the elevation of T occurred (Meulenberg & Hofman, 1991; Klinga et al., 1978).

In sum, no support was found in this study for a maternal–fetal route of the fetal hormone transfer notion. It seems therefore likely that the maternal compartment of the maternal–fetal unit is a fairly different one than the fetal compartment (i.e., the amnion sac). It has been shown rather convincingly in several studies (e.g., Nagamani et al., 1979; Rodeck et al., 1985; van de Beek et al., 2004) that amniotic fluid T levels are higher when measured in the presence of a male fetus compared to a female fetus. This supports the notion that prenatal exposure to T in fetuses is not so much via the more indirect maternal–fetal route as the fetal hormone transfer notion suggests, but rather via the other route, that is, the feto–fetal route. In animal research, this mechanism has been observed more systematically and there is clear support that transport of T occurs through fetal membranes (e.g., Even et al., 1992).

However, since a change was observed in several maternal serum steroids during gestation which is assumed to be of fetal origin no matter which sex (Bammann et al., 1980), any exchange of steroids between the maternal–fetal compartment can not be completely ruled out. Evidence for such an underlying assumption, that fetal and maternal compartments affect each other's hormonal states, is mentioned in the literature (see Miller, 1998).

Although the participating pregnant women were not selected on the basis of the sex of their unborn children, the number of women pregnant with a MM twin-pair turned out to be unexpectedly low (9) compared to the other types of twin pregnancies (29 OS and 17 FF twins). This may be due to an artifact resulting from hormone-induced pregnancies (James, 1986; Orlebeke et al., 1993). However, this does not seem to be the case in our population; see Table 1.

In summary, we must be cautious in drawing any firm conclusions since it is possible that we were not able to demonstrate an effect of fetal sex on maternal serum steroid levels due to the fact that one of the subgroups (i.e., the MM group) was small.

However, the results seem to suggest for now that any presumed increased prenatal exposure to T in female fetuses sharing the womb with males, as in opposite-sex twins, occurs mainly through the direct feto–fetal route of hormone transfer in the amniotic sac and not indirectly via the maternal–fetal unit. Since this study failed to find evidence for the mater-

nal–fetal (indirect) route, we believe that the two routes of hormone transfer are dependent on different compartments. Measuring maternal serum steroid levels in the maternal unit during pregnancy does not seem to be of any use in investigating prenatal exposure to steroids in twins.

Nevertheless, final conclusions can only be drawn after these results have been replicated.

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