Running head: FETAL GLUCOCORTICOID SYNTHESIS

Full-term deliveries without antecedent labor reveal sex differences in umbilical cord glucocorticoid concentrations

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Abstract

Background: Previous studies have shown that pregnant women have higher salivary cortisol levels when the fetus is female. These findings suggest a basis for the sex differences observed in many offspring outcomes after exposure to *in utero* stress, but it is not known if fetal adrenal glucocorticoid synthesis differs by sex.

Methods: Arterial and venous umbilical cord blood samples were collected immediately after scheduled cesarean delivery (n = 52, 25 female). Cortisol and corticosterone concentrations were quantified by liquid chromatography coupled to tandem mass spectrometry.

Results: Sex differences were observed for fetal arterial and venous cortisol and venous corticosterone, with higher levels present when the fetus was female. However, sex differences were not observed for fetal synthesis of cortisol, suggesting that the fetus does not control the differences observed in cord blood glucocorticoids.

Conclusions: The presence of sex differences in umbilical cord glucocorticoid concentrations in the absence of sex differences in glucocorticoid synthesis by the fetal adrenal gland suggests that these differences have a maternal or placental origin. Thus, the *in utero* glucocorticoids in circulation are sex-specific and may have developmental importance for sex differences in psychiatric and neurodevelopment disorders that display sex biases.

Sex differences within the intrauterine milieu have profound and enduring implications for fetal and child development (Clifton, 2010; Sandman, Glynn, & Davis, 2013). For example, in the presence of maternal asthma, female fetuses reduce their growth whereas males do not (Murphy et al., 2003). However, if the mother experiences an acute exacerbation of asthma, males have a greatly increased incidence of intrauterine growth retardation, preterm delivery and stillbirth (Clark et al., 2007; Murphy, Gibson, Talbot, & Clifton, 2005). In addition, sex differences in neurodevelopmental and behavioral outcomes are observed following intrauterine exposure to stress and stress hormones (Sandman et al., 2013), suggesting that sex differences within the intrauterine environment may be linked to sex differences in neurodevelopmental and psychiatric disorders (Davis & Pfaff, 2014). Given that glucocorticoid receptors are highly expressed in the fetal brain (Sanchez, Young, Plotsky, & Insel, 2000), affecting the expression of more than one-thousand genes (Salaria et al., 2006), sex differences in prenatal cortisol exposure have the potential to shape many aspects of brain development.

Maternal adaptation to pregnancy can alter the developmental environment of the fetus in response to fetal sex. For example, women pregnant with a female fetus had flatter daytime salivary cortisol slopes compared to women pregnant with a male (Giesbrecht, Campbell, Letourneau, & Team, 2015), and mean salivary cortisol levels were also higher in women pregnant with a female during the last weeks of pregnancy (DiPietro, Costigan, Kivlighan, Chen, & Laudenslager, 2011). It has, however, been challenging to determine whether the two sexes differentially alter their own developmental environments.

The intrauterine glucocorticoid milieu is complex, receiving contributions from the placenta (which is steroidogenic) and from both maternal and fetal circulation. In addition to producing steroid hormones, the placenta also expresses high levels of the enzyme 11ß hydroxy-

steroid dehydrogenase (11ßHSD) that converts both cortisol and corticosterone to cortisone and 11-dehydrocorticosterone, respectively (White, Mune, & Agarwal, 1997). This conversion of glucocorticoids from biologically active to inactive forms is thought to limit fetal exposure to maternal glucocorticoids transferred into placental circulation (Benediktsson, Calder, Edwards, & Seckl, 1997). By approximately mid gestation the fetal adrenals begin synthesizing glucocorticoids (Ishimoto & Jaffe, 2011) and this synthesis can be observed in the increase in umbilical glucocorticoid concentrations from venous (circulation toward the fetus from the placenta) to arterial (circulation from the fetus toward the placenta) circulation (Wynne-Edwards, Edwards, & Hancock, 2013). Here we focus on umbilical circulation to address questions about fetal exposure and glucocorticoid synthesis.

Elective Caesarian sections offer an extraordinary window into full-term fetal stress steroid exposure and synthesis. The mother presents at the delivery site without going into labor, and without acute clinical indications of distress in the mother or fetus that require emergency intervention. In a previous sample of 10 elective Caesarian sections for which mixed cord blood (predominantly venous blood) was collected, there was no evidence of a sex difference in cortisol concentration (Clifton, Bisits, & Zarzycki, 2007).

A larger sample of 265 deliveries (Wynne-Edwards et al., 2013), of which 53 were elective Caesarian deliveries, determined both cortisol and corticosterone concentrations for the umbilical vein and artery. Our previous study demonstrated that the full-term fetus synthesizes both cortisol and corticosterone, and, specifically, that fetal corticosterone synthesis, rather than cortisol synthesis, was associated with fetal distress (Wynne-Edwards et al., 2013). However, sex of the fetus was excluded as an analytic covariate in those analyses before focusing on the subset of participants who underwent elective Caesarian section without antecedent labor, and therefore

without elevation in maternal or fetal glucocorticoids consequent to labor. The current analysis re-visited the data from that study to test the hypothesis that fetal sex would influence fetal exposure and glucocorticoid synthesis. Maternal-fetal communication operates bi-directionally via both cortisol and corticosterone, and under conditions of stress the mother preferentially secretes cortisol (Cawson, Anderson, Turnbull, & Lampe, 1974) whereas the fetus preferentially secretes corticosterone (Wynne-Edwards et al., 2013). Accordingly, we set out to determine if concentrations of cortisol or corticosterone in cord blood are sexually dimorphic and if fetal synthesis differs for males and females. We reasoned that sex differences in umbilical corticosterone would suggest fetal control whereas sex difference in cortisol would suggest a role for the materno-placental unit.

Methods

Full methods are available in Wynne-Edwards et al., 2013. Within a sample of 265 healthy, full-term (>37 completed weeks), singleton births, the subset of 53 women with elective Caesarian section without antecedent labor were included in this analysis. Race/ ethnicity and socioeconomic status were not assessed, however the catchment area for the participating hospital was predominately white, middle class and suburban, and likely underrepresents a broad diversity of races/ethnicities and socioeconomic levels. Written consent was obtained from participants upon arrival at the labor and delivery unit, and covered only the maternal birth record. Chart data for the mother and child were not examined. The study was approved by the University of Calgary Conjoint Health Research Ethics Board.

At the research site, standard postpartum protocol routinely samples umbilical arterial and venous blood separately for pH and acid-base status. After that routine sampling, but prior to delivery of the placenta, one non-heparinized, ‘red top’, vial of venous and another of arterial

whole blood were collected by needle aspiration (16 gauge) for this study. Samples were immediately refrigerated, and allowed to stand for at least one hour (range 1–12 h, median < 4 h), before being centrifuged (4000 G for 5 min). Serum was separated and stored at -20°C until delivery to the research laboratory for hormone analysis.

Detailed methods for simultaneous quantitation of cortisol and corticosterone concentrations by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) have been previously reported (Koren, Ng, Soma, & Wynne-Edwards, 2012; Wynne-Edwards et al., 2013). Briefly, cortisol and corticosterone concentrations were determined as area ratios relative to a bio-identical deuterated internal standard. Intra- and inter-assay coefficients of variation were 4.5% and 2.9% for cortisol, and 5.8% and 3.6% for corticosterone. No samples fell outside of the linear range of the calibration curve (Wynne-Edwards et al., 2013).

Sex differences were assessed through three parameters: 1) the concentration of corticosteroid (ng/ml) in venous and arterial circulation; 2) the increase (ng/mL) in concentration between venous and arterial circulation (i.e., increase in concentration due to fetal synthesis); and 3) the proportional change across the fetus that considers the fetal synthesis as a proportion of the arriving concentration ((arterial-venous)/venous)). Comparisons between the sexes used a t-test with degrees of freedom adjusted for unequal variances. All statistical tests used JMP 12.1.0 (www.jmp.com) and applied a critical alpha of .05. Effect sizes were estimated with Cohen's *d*. Effects greater than $d = .41$ are, by convention, considered clinically significant (Ferguson, 2009).

Results

One female fetus had an arterial cortisol value of 253 ng/ml, which was an outlier relative to the sample median of 35 ng/ml (range 8 – 125 ng/ml; SD 30.5). Recent antenatal

glucocorticoid administration to the mother was suspected. That female fetus was excluded, resulting in a final sample size of 25 females and 27 males.

As expected, common antecedent risk factors for this sample were previous Caesarian section (n=38, 73%, 19 F, 19 M) and maternal age over 35 (n=22, 42%, 10 F, 12 M). Both factors contribute to physician decisions to schedule an elective section. Other antecedent risk factors (e.g., gestational diabetes or hypertension) were too rare to be analyzed within this cohort. Also as expected, there were sex differences in birthweight, with females smaller than males (Table 1, $p = 0.05$).

Sex differences in glucocorticoids in the umbilical circulation

Females had higher levels of cortisol than males in both venous (travelling from the placenta towards the fetus) and arterial (travelling from the fetus towards the placenta) umbilical cord serum (Table 2). Differences for corticosterone were in the same direction, but not significant. Thus, cortisol levels were higher in cord blood circulation among females compared to males.

Sex differences in fetal adrenal glucocorticoid synthesis

Cortisol and corticosterone concentrations both increased from venous to arterial circulation (arterial minus venous) confirming synthesis by the fetal adrenal, as was previously reported (Wynne-Edwards et al., 2013). However, fetal synthesis of cortisol and corticosterone did not differ by sex (Table 2). Proportional increases ((arterial-venous)/venous) also failed to detect a sex difference in fetal synthesis of cortisol. Thus, there was no evidence that fetal adrenal glucocorticoid synthesis was differentiated by fetal sex.

Discussion

Arterial and venous umbilical cord blood samples collected immediately after scheduled cesarean delivery were used to represent the physiological concentrations of cortisol and corticosterone in the full-term fetal circulation by avoiding the confounding influence of maternal glucocorticoid increases caused by labor. Sex differences were observed for fetal arterial and venous cortisol, with higher levels of cortisol present when the fetus was female (Figure 1). Higher cortisol concentration in female arterial and venous umbilical cord blood is consistent with findings of higher salivary cortisol levels in maternal circulation during late pregnancy (DiPietro et al., 2011; Giesbrecht et al., 2015) and higher cortisol in female mixed umbilical cord blood following antenatal betamethasone exposure (Stark, Wright, & Clifton, 2009).

Sex differences were not observed for fetal synthesis of cortisol or corticosterone, suggesting that sex differences in umbilical circulation are not from the fetus itself. Male and female fetuses both added cortisol and corticosterone to the umbilical circulation, as was shown previously (Wynne-Edwards et al., 2013). However, the absolute increases and the proportional increases (relative to levels arriving at the fetus) did not differ by sex. Thus, while females experienced higher cortisol concentrations overall in the umbilical circulation, sex-specific glucocorticoid synthesis did not contribute to that sex difference (Figure 1).

The absence of sex differences in glucocorticoid synthesis by the fetus supports a maternal and/or placental role in determining the higher circulating concentrations of cortisol when the fetus is female. Women carrying a female fetus exhibit higher salivary cortisol levels and blunted diurnal patterns of salivary cortisol during late pregnancy (DiPietro et al., 2011; Giesbrecht et al., 2015). Higher levels of salivary cortisol in women pregnant with a female fetus

and higher levels of umbilical cord cortisol in the female fetus highlight the potential importance of the intimate interaction between maternal circulation and feto-placental circulation (Figure 1).

Sex differences in maternal circulation might play a direct role in the sex differences observed in fetal umbilical circulation. Conversely, sex differences in umbilical circulation could also be driving sex differences in maternal cortisol concentrations. In addition, there is potential for sex-specific modification of glucocorticoid concentrations in umbilical circulation by placental 11ßHSD. For example, among term-born fetuses of normal weight, placental 11ßHSD expression is lower when the fetus is female, suggesting less placental inactivation of cortisol (conversion to cortisone) by the female fetus (Mericq et al., 2009) which could be the basis for the overall elevation of umbilical cord cortisol when the fetus is female. However, following betamethasone exposure, placental 11ßHSD is expressed at higher levels when the fetus is female, suggesting a greater responsiveness of the female fetus to external perturbations of the HPA axis (Stark et al., 2009) and the potential for improved protection of the full-term female fetus from maternal cortisol released during labor. These findings suggest that the female fetus is more sensitive to *in utero* cortisol exposures – a conclusion that was first made with regard to fetal exposure to maternal asthma (Murphy et al., 2003).

In addition, the tissue-specific expression of glucocorticoid and mineralocorticoid receptors, and the circulating concentrations of glucocorticoid binding proteins (corticosteroid binding globulins and albumin) have the potential to play an important role in determining the circulating concentrations and could be regulated in sex-specific ways that alter the fetal responses to the glucocorticoid (Clifton, 2010). In preterm placentae, for example, expression of glucocorticoid receptors differs as a function of fetal sex, and at least with regard to females, glucocorticoid receptors are related to regulation of fetal growth (Saif et al., 2015). Thus,

although the mechanisms producing sex-specific differences in umbilical glucocorticoids are not yet known, it is reasonable to predict that these sex differences are developmentally important.

Clinically, these findings may have implications for our understanding of sex differences in the development of the fetal HPA axis and subsequently to sex differences in stress-related disorders (e.g., depression). Evidence that early-life events program physiological responses to stress later in life is now widespread (Mina & Reynolds, 2014). Sex differences in the levels of umbilical glucocorticoids are a potential mechanism by which maternal stress during pregnancy leads to sex-specific effects on infant HPA axis function (Giesbrecht, Letourneau, Campbell, & Team, 2016). Furthermore, such sex differences in prenatal exposures and infant HPA axis function may contribute to sex differences in the prevalence and presentation of some neurodevelopmental and psychiatric disorders (Sandman et al., 2013).

Similar findings have been reported in mice - a species where corticosterone is the dominant glucocorticoid. Specifically, female fetal mice delivered by caesarian section at term had higher corticosterone concentrations in trunk blood than male fetuses. (Montano, Wang, & vom Saal, 1993). That study also looked at fetal adrenal corticosterone synthesis and found no sex difference in the capacity of the fetal mouse adrenal glands to synthesize corticosterone. (Montano, Wang, & vom Saal, 1993). Their conclusion was similar to the current study. More of the dominant glucocorticoid was present when the fetus was female, relative to when it was male, and materno-placental mechanisms, rather than fetal synthesis and clearance, were implicated.

Strengths and Limitations

Data for this study was based upon the birth record and samples collected at birth and as such does not account for potential confounders, such as smoking, which may affect umbilical

glucocorticoid levels (Varvarigou, Petsali, Vassilakos, & Beratis, 2006), and potential effect modifiers, such as SES or race/ethnicity. Interpretation of these findings should bear in mind that the sample was obtained from a hospital serving a mostly middle class, white, mature, and urban population. Despite the small sample size, this is the largest study (that we are aware of) to assess umbilical glucocorticoids in cesarean deliveries and the first to show that fetal synthesis of cortisol is not a likely source of sex differences in umbilical circulation.

Conclusion

In caesarian births without antecedent labor, the umbilical circulation for a female fetus contained higher concentrations of cortisol than the umbilical circulation for a male fetus. There was no evidence, however, of a sex difference in fetal synthesis of cortisol, thus we infer that the fetal adrenal gland is not directly responsible for this sex difference. These finding focuses future research towards the placental side of the umbilical circulation where maternal, fetal and/or placental biology may interact to generate sex difference in umbilical circulation.

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Table 1. Sample characteristics.

	Females (n = 25)		Males (n = 27)		
	Mean	SE	Mean	SE	*p*
Maternal age (years)	33.48	0.69	32.63	0.86	.44
Parity	1.04	0.12	1.22	0.20	.45
APGAR at 1 minute	8.52	0.22	8.33	0.24	.57
Birthweight (g)	3307	91	3596	114	**.05**

Note: *p* values refer to t-test comparisons between males and females.

Table 2. Sex differences in mean (SE) glucocorticoid concentrations in venous and arterial umbilical circulation, glucocorticoid increases (Arterial-Venous), and proportional increases ((Arterial-Venous)/Venous).

	Cortisol				Corticosterone			
	Female	Male	t-Test	Cohen's *d*	Female	Male	t-Test	Cohen's *d*
Venous (ng/mL)	33.82 (4.43)	21.52 (2.03)	2.52, *p* = **.02**	0.71	1.43 (.17)	1.06 (.09)	1.91, *p* = .06	0.53
Arterial (ng/mL)	52.84 (7.03)	34.80 (4.40)	2.17, *p* = **.04**	0.61	4.01 (.63)	3.05 (.57)	1.12, *p* = .27	0.31
Arterial-Venous	19.02 (3.28)	13.28 (2.65)	1.36, *p* = .18	.38	2.58 (.53)	1.99 (.51)	.80, p = .42	.22
(Arterial-Venous)/Venous	.52 (.07)	.53 (.07)	.05, *p* = .96	.01	1.75 (.34)	1.72 (.31)	.08, *p* = .94	.02

Figure Legend

Figure 1. Schematic representation of sex difference found in umbilical cord cortisol from full-term deliveries by elective Caesarian section without antecedent labor. The male circulation is represented by black arrows and the female by white arrows. In the bottom-center of the figure, in the umbilical circulation compartment, the concentration of cortisol in male venous circulation is set to 1.0 arbitrarily. Venous umbilical cortisol in the female is higher (= wider arrow), at 1.57 based on the female/male ratio. At the right hand side, fetal adrenal cortisol synthesis increases the cortisol concentration reaching the umbilical arterial circulation by 1.6x (arrow width increases) in both male and female fetuses. In the top-center, the male arterial cortisol is again set to 1.0 (although now increased over the venous concentration), demonstrating that the female/male ratio is similar (1.52 ratio). At the left hand side, the placental circulation is represented. Cortisol is converted to cortisone through the action of the 11ß hydroxy-steroid dehydrogenase enzyme (11ßHSD), reducing cortisol concentration entering the venous circulation. At the same time, maternal circulation is exchanging cortisol with the placental compartment with unknown (?), potentially sex-specific, impacts on maternal, placental and umbilical cord cortisol concentrations (male and female pregnancies represented by dark and light stippled arrows).