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Sex-Specific Effects of Prenatal Glucocorticoids on Placental Development

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1. Introduction

The placenta, essential for normal fetal development by providing adequate nutrients to allow appropriate growth and maturation of the fetus in preparation for birth, is also a 'protective' barrier in the sense that it prevents entry into the fetal circulation of substances that are either toxic, or that drive fetal growth at inappropriate rates. An important aspect of this 'filtering' function of the placenta is limiting the entry of glucocorticoids of maternal origin into the fetal compartment. This is achieved by the presence of enzymes, transporters and receptors collectively termed the 'placental glucocorticoid barrier' [1-4].

Antenatal glucocorticoids are routinely administered to the mother for the treatment of a variety of pregnancy and fetal complications. Asthmatic women often experience an increase in severity of their symptoms during pregnancy leading to increased use of glucocorticoids. The threat of preterm birth results in administration of the synthetic glucocorticoid, betamethasone, to rapidly mature fetal organs (especially, the lungs) to promote survival. Further, stressful events during pregnancy such as natural disasters and famines for example, expose fetuses to higher than normal levels of maternally secreted glucocorticoids.

The effects of exposure to high levels of glucocorticoids during fetal development have now been well described [1, 5-9], and the advantages (e.g., maturation of lung surfactant production and increased hepatic glycogen deposition) are offset by effects that limit fetal growth and induce perturbations of brain growth and perfusion [1, 10-11]. However, while fetal/neonatal effects have been intensively investigated, the consequences of glucocorticoid excess on placental structure and function has received little attention to date. The knowledge that male fetuses are more likely to be affected negatively following events that usually increase fetal glucocorticoid exposure, has alerted researchers to the possibility that such sex-related effects could arise in the placenta. This chapter will describe the differences

that exist between a male and female placenta with respect to the glucocorticoid barrier, and summarise current human clinical and experimental animal work that has explored the differential response of the placenta of a male and female fetus to glucocorticoid exposure.

2. The placental glucocorticoid barrier

While glucocorticoids are essential for the development of many organs, during pregnancy, the placenta acts as a barrier to prevent excess entry of maternal glucocorticoids into the fetal compartment [12-14]. This placental barrier to glucocorticoids is achieved predominantly by the presence of 11 β -hydroxysteroid dehydrogenase type 2 (11 β HSD2), which converts the biologically active glucocorticoid (cortisol in humans, corticosterone in mice and rats) to its physiologically inert form [2]. The placental 'barrier' is not complete, and under normal conditions a proportion (~10-15%) of maternal glucocorticoids reaches the fetal circulation [2]. While 11 β HSD2 is the major component of the placental glucocorticoid barrier, other proteins contained within the placenta may also help to limit the transfer of maternal glucocorticoid to the fetus. The multi-drug resistance P-glycoprotein (ABCB1) is a membrane-bound protein, which mediates the efflux of glucocorticoids out of the placenta back into the maternal circulation, thus reducing the amount of glucocorticoids able to diffuse down the concentration gradient into the fetal circulation [15-17].

The response of the placenta itself to glucocorticoids is mediated by the glucocorticoid (GR) and mineralocorticoid receptor [18]. The most prominent isoform of the GR, both in the placenta and throughout the whole body, is GR α . This isoform mediates the biological effects of glucocorticoids, which include cell growth, proliferation and differentiation [19]. The placenta has not generally been considered a mineralocorticoid target tissue, however work by Driver et al [20] has suggested that placental trophoblast cells express a functional mineralocorticoid receptor, which is in part responsible for the transport of sodium across the placenta [20]. Because of the limited data on the role of the mineralocorticoid receptor in the placenta, our discussion will focus primarily on GR mediated effects.

3. Causes of elevated glucocorticoids during pregnancy

There are many circumstances during pregnancy in which the circulating levels of maternal glucocorticoids are elevated, resulting in placental and fetal exposure to excess glucocorticoids. The glucocorticoids within the maternal system can either be endogenous, originating from within the mother; or exogenous, where the glucocorticoid has been administered to the mother as a drug or treatment. Exogenous glucocorticoids are generally synthetic, such as betamethasone, dexamethasone or prednisone. The period of time when maternal, and therefore fetal and placental glucocorticoid levels, are elevated will vary considerably depending on the clinical circumstance, and effects arising from either acute or chronic exposures have been identified. Thus, the type of glucocorticoid, duration of exposure, and time in gestation need to be taken into account when determining the consequences for the fetus and placenta.

3.1. Exposure to natural glucocorticoids

Periods of stress, both physical (illness, excess exercise, famine/under nutrition) or psychological (anxiety) in origin, result in the elevation of endogenous glucocorticoids [21]. While cortisol can cross the placenta, it is a good substrate for 11β HSD2, and is readily catalysed by this enzyme under normal levels. However, when levels of cortisol are elevated, the barrier is overwhelmed and more cortisol is able to cross the placenta into the fetal circulation [4]. Deleterious effects of excess endogenous glucocorticoids on the fetus and newborn have been well documented [3, 6, 22-25]. These effects are greater for male fetuses. For example, males have been shown to have greater instances of *in utero* mortality and also increased likelihood of childhood and adult morbidity and mortality [21, 26]. An epidemiology study that examined women who were pregnant at the time of the 2001 terrorist attacks on the World Trade Centre found that there was a higher incidence of low birth weight babies for women who were residing in New York at the time. The greatest proportion of these low birth weight babies came from women who were in their first or second trimester at the time of the attacks [27]. These studies also revealed an increased incidence of male fetal death in New York after September 2001 [28-29]. High maternal stress and thus fetal exposure of cortisol are thought to be the cause of these poor fetal outcomes.

3.2. Exposure to synthetic glucocorticoids

Antenatal glucocorticoids are routinely administered to the mother for the treatment of a variety of pregnancy and fetal complications. Women who suffer from asthma are required to continue their glucocorticoid medication for the ongoing treatment/prevention of their symptoms, which in 33% of cases worsen during pregnancy [30-31]. Women whose babies are at risk of congenital adrenal hyperplasia are administered antenatal glucocorticoid treatment to return fetal adrenal hormone levels to normal and thus virilisation (the abnormal development of male sexual characteristics in a female) and fertility problems are prevented [32]. Further, pregnant mothers threatening preterm birth (~7-10% of all pregnancies), receive antenatal glucocorticoids, to mature the lungs of the fetus prior to birth to reduce neonatal morbidity and mortality [25]. As for cortisol, synthetic glucocorticoids can be catalysed by 11β HSD2, however they are a poor substrate for the enzyme, and more freely cross the placenta than cortisol [33]. The presence of excess maternal glucocorticoids can have positive effects on fetal development and maternal health and in many situations cannot be avoided. The National Institute of Health recommend treatment of all women at risk of preterm delivery, between 24 and 34 weeks of gestation, with synthetic glucocorticoids to prematurely mature fetal organs, primarily the lung, to improve neonatal survival [8]. Therefore a large proportion of this population of babies, are exposed to single, and sometimes multiple courses of synthetic glucocorticoids in the period leading up to birth [25]. While antenatal glucocorticoids are the most effective treatment for improving preterm birth survival rates, the scientific community continues to question whether the use of glucocorticoids to reduce the morbidity and mortality associated with preterm birth, is worth the risk of the potential negative outcomes on metabolism and

neurodevelopment seen within these babies during childhood and into adulthood [9]. While the consensus is currently 'yes', the guidelines for women threatening preterm birth state that only a single, and not multiple doses of glucocorticoids, should be given until more convincing data of the benefits of multiple doses are obtained [34]. Much work is examining the outcomes of antenatal glucocorticoids for the fetus, however the effect of glucocorticoids on the placenta, including the potential sex-specific effects, need to be considered as these may contribute to, or compound, the fetal outcomes.

4. Excess glucocorticoids and the programming of disease

The Barker Hypothesis of Developmental Origins of Health and Disease (DoHaD) states that diseases, such as coronary heart disease, hypertension and diabetes, may be consequences of *in utero* 'programming', whereby a stimulus or insult at a critical, sensitive 'window' of fetal life results in long-term changes in structure, physiology or metabolism, leading to diseases later in life [35]. These stimuli are likely to be mediated via a number of different hormones and immune factors, but glucocorticoids have been singled out as one of the most prevalent factors. Epidemiological and experimental animal studies have revealed that excess glucocorticoids *in utero* can be linked to the development of diseases such as hypertension [36-42], depression [43], cardiovascular disease [44], diabetes [45-46] and attention deficit disorders [23, 47]. Further, the outcomes or the severity of these diseases are worse if the offspring affected are male [37-39, 48-51].

Recently, a role for the placenta in mediating developmental programming of excess glucocorticoids and other *in utero* events has been suggested [44, 47, 52-55]. The size (both absolute and relative to fetal size), shape, and vascular development of the placenta have all been identified as potential predictors of adult onset diseases. For instance, a small baby with a large placenta has a relative risk of adult hypertension 3 times that of a large baby with a normal placental size [44]. Further, the abolition of a gene vital for placental, but not cardiac vascular development (*HOXA13*) has been shown to be embryonic lethal in mice, indicating that placental hemodynamics play an important role in the development of the heart, and alterations may lead to the development of cardiovascular problems later in life [56].

5. Susceptibility of the placenta to negative outcomes from glucocorticoid exposure

There are several reasons for the susceptibility of the placenta to adverse outcomes caused by excess glucocorticoid exposure. I.) The placenta is in direct contact with the maternal circulation and thus is directly bathed in circulating maternal glucocorticoids. II.) One of the main roles of the placenta, as described above, is as a barrier to prevent fetal exposure to excess glucocorticoids; therefore the placenta may be directly altered by the glucocorticoid exposure *before* 11 β HSD2 is able to convert these to their inactive metabolites [57]. III.) The structural development of the placenta occurs through a series of branching events, particularly the placental vasculature. This branching occurs similarly in other organs, such as the lung and kidney, which are particularly vulnerable to excess glucocorticoid exposure during periods of

extensive branching [58-60]. Indeed in the evolution of the placenta, the genetic pathways that regulate branching morphogenesis in these other organs has been utilised by the placenta [61-65]. Hence a similar susceptibility could be expected for the placenta.

6. Sex-specific placental regulation of glucocorticoids

The placenta is primarily derived from embryonic tissue and therefore has the same genetic content as the fetus. In recent studies examining both human and animal models, a number of fundamental differences between the placenta of a male and female fetus have been uncovered. Differences in placental proportions [66] and surface area [67] have been noted in placentas of males and females. Specifically, females have been shown to have a greater exchange region of the placenta compared to males [66], and within this exchange region, females have been reported to have a larger surface area [67]. Expression of genes and proteins known to have fundamental roles in controlling placental development [66], nutrient transfer, and other placental functions [68-71] differ between a male and female placenta. Specifically, placentas of male fetuses have been reported to have higher levels of the glucose transporter [66], higher levels of epidermal growth factor binding protein at term [68], but lower levels of activity of the sodium-hydrogen exchanger [70]. Levels of pregnancy hormones, produced by the placenta, differ for a male and female. For example, maternal serum human chorionic gonadotrophin levels are significantly higher in pregnancies carrying a female fetus from as early as 3 weeks of pregnancy [72]. Placental levels of progesterone also differ for a male and female fetus in many species, with a study in the gray seal, for example, showing higher levels in females than males [73]. Because progesterone is primarily of placental origin, these differences provide further evidence of the fundamental differences that exist in the placenta of a male and female fetus. Further, the placenta of a male and female fetus have also been shown to respond differently to adverse *in utero* environments including maternal under-nutrition/famine [74] and *in utero* infection [75]. For example, female placentas demonstrated more striking alterations in gene expression in response to restrictions in maternal diet than male placentas when examined by microarray analysis. Further, placentas of male fetuses exhibited a greater immunological reaction (greater expression of TNF α , IL-10, and PTGS2) to simulated *in utero* infection. Whether these responses differ because of the fundamental differences that exist between the sexes remains unknown.

Normal physiological glucocorticoid levels: During pregnancy, the term female placenta has significantly higher expression of the GR [31] and 11 β HSD2 activity [76] than placentas of male fetuses. Glucocorticoids are known to negatively regulate GR expression [66, 77], therefore the higher GR expression within placentas of female fetuses may be physiological evidence that the female fetal-placental unit is exposed to less bioactive cortisol at term than the male. A downstream consequence of lower glucocorticoid levels may be an enhanced immune response. The activation of the fetal immune system is associated with the activation of the fetal hypothalamic pituitary adrenal axis, which results in the production of glucocorticoids, which in turn modulate the inflammatory response. Glucocorticoids function in a negative feedback loop with the hypothalamic pituitary adrenal axis, such that high glucocorticoid levels suppressing immune function [78]. It has been suggested that this

may contribute to the increased viability of female fetuses exposed to a sub-optimal *in utero* environment, compared to males, who are particularly vulnerable to changes in the maternal environment in which increased levels of glucocorticoids are often seen [79] (see above).

Response to excess glucocorticoids: The adverse effects of glucocorticoids during pregnancy on placental weight in the human have been reported as early as 1977 by Koppe and others [80]. Since then, a number of studies, in both humans and animal models, have demonstrated that excess glucocorticoids during gestation have a wide range of consequences for the placenta, which impact its structure and function, ultimately impacting the fetus [22, 81-91]. Recently, these consequences have been shown to occur in a sex-specific manner.

Human evidence

Evidence is beginning to emerge from studies in the clinical setting demonstrating that human placentas are sexually dimorphic in their regulation of normal glucocorticoid levels and these differences are exacerbated in response to excess maternal glucocorticoids. Much of this clinical evidence is arising from the work of Clifton and colleagues, who focus on identifying the effect of glucocorticoids on fetal and placental development, by studying mothers who suffer from asthma and thus use inhaled glucocorticoid treatments throughout their pregnancy. Asthma affects between 3% and 12% of pregnant women worldwide and the prevalence among pregnant women is rising [92]. It is well recognised that women (and their babies) with asthma are at increased risk of poor pregnancy outcomes [93]. Clifton and colleagues have also examined preterm babies and the consequences of excess glucocorticoid exposure on their placentas.

6.1. Effect of glucocorticoids on placental development and other pathways

Female babies born to asthmatic mothers, who utilised inhaled glucocorticoid treatments to manage their symptoms, were found to be growth-reduced unlike male babies born to asthmatic mothers, who were normally grown, despite similar cord blood cortisol levels [76]. Placentas of male and female babies born to these mothers, had reduced vascularisation within the placental villi, resulting in reduced absolute fetal capillary volume [94], although this was most striking in placentas of male fetuses. Further, placentas of glucocorticoid-exposed males also had a reduced fetal capillary length [94], which was not observed in placentas of female fetuses. The authors speculate that glucocorticoid treatment may adversely affect placental vasculogenesis and/or angiogenesis by causing endothelial cell rounding and capillary regression, an observation made in other tissues after glucocorticoid exposure [95-97]. These effects may be mediated by members of the vascular endothelial growth factor family or inflammatory cytokines, both of which play a key role in placental vasculogenesis and angiogenesis [98]. The observed changes in placental morphometry in male placentas would be expected to affect placental hemodynamics, however the absence of these changes in the female placenta do not adequately explain the reduced fetal growth of the female fetus in this high glucocorticoid environment.

A study by Stark et al [99], examined the placental pro-: anti-oxidant balance in response to antenatal betamethasone in placentas of preterm babies. Glucocorticoids have previously been shown to influence fetal reactive oxygen species production and antioxidant defences [100-101]. These pathways are involved in preparing the fetus for the increase in free oxygen radical generation which is experienced during the fetal to neonatal transition [102]. Stark and colleagues observed that a pro-oxidant state was present in placentas of male fetuses, but not females following glucocorticoid exposure. Specifically, they reported that males had higher levels of the oxidative stress marker, protein carbonyl and a decreased level of the anti-oxidant enzyme, glutathione peroxidase. The authors suggested that these findings could contribute to the patho-physiologic processes underlying oxygen radical diseases of the newborn [99]; conditions known to exhibit a male excess [103].

6.2. Sex-specific effects of excess glucocorticoids on the placental glucocorticoid barrier

As the placental glucocorticoid barrier demonstrates sexually dimorphic regulation under normal conditions, and the placental response to glucocorticoids is crucial in determining fetal growth outcomes, the effect of excess maternal glucocorticoids on this barrier have been investigated. The expression of the GR within the placenta of male and female fetuses is reported to be sexually dimorphic under normal conditions (females having higher expression levels), whereas the response of GR to excess glucocorticoids is similar between the sexes [77].

In preterm babies, whose mothers received antenatal betamethasone, the activity of placental 11 β HSD2 (predominant component of the glucocorticoid barrier) was reduced in placentas of male fetuses only [104]. This reduction in 11 β HSD2 activity would be expected to compound the already increased exposure of the male fetus to cortisol brought about by the decreased term 11 β HSD2 activity within male placentas during a normal pregnancy [76]. This may further compound the reduced immune function in male fetuses, thus increasing their susceptibility to disease. Further, glucocorticoids are important for fetal adrenal development [104]. Male preterm babies exposed to excess glucocorticoids *in utero*, have less adrenal activity than female preterm babies exposed to similar levels of glucocorticoids [104], which may explain the increased risk of morbidity and mortality of preterm male babies. We are unaware of any studies that have investigated the other members of the placental glucocorticoid barrier (MR and ABCB1) and their sexually dimorphic response to excess glucocorticoid exposure.

Animal models of glucocorticoid exposure

The effects of excess maternal glucocorticoid exposure, on placental growth and development, has been investigated using a range of animal models including the sheep [105-106], rat [85], mouse [107] and spiny mouse [66]. Most of these studies have utilised synthetic glucocorticoids (namely dexamethasone or betamethasone) and been designed to mimic the level of exposure experienced by the preterm infant. However, there are also a

large number of studies using glucocorticoids at other developmental time points including very early in gestation. When considering the data generated from animal models, it is important to take into consideration not only the timing of glucocorticoid exposure but also the timing of placental and fetal development in the species being used, as there is considerable variation in placental development and overall structure between species. Many of these studies, particularly those in the sheep and rat, have not analysed data according to fetal sex. However a couple of recent studies in the mouse and spiny mouse have demonstrated markedly different outcomes in placental development and gene expression in placentas of males and females suggesting that alterations occurring within the placenta, following glucocorticoid exposure, are dependent upon fetal sex.

Sheep

The sheep placenta is made up of 60-70 individual placentomes called cotyledons, which are cup shaped structures with fetal tissue surrounded by maternal tissue. Administration of dexamethasone for 48 hours between 64-66 days of gestation (term=145-150 days) resulted in generally larger cotyledons with overgrowth of the fetal tissue when the placenta was examined at completion of the infusion [105]. However, this was not observed in other studies using betamethasone later in gestation (around 100 days of gestation) [108]. Unfortunately, neither of these studies separated data according to fetal sex. In another study, pregnant ewes received intramuscular injections of dexamethasone on day 40 and 41 of gestation and the placentas were examined at day 50, 100 or 140 days of gestation. In this case, data was analysed separately for males and females and whilst dexamethasone exposure significantly increased placental *11 β HSD2* mRNA levels in males compared with controls at 50 and 140 days, in female placentas, levels were not altered by the dexamethasone exposure [106].

Rat

Dexamethasone exposure during late pregnancy has been shown to significantly reduce placental weight in the rat [91, 109]. This was associated with reduced expression of vascular endothelial growth factors (*VEGF*) and placental vascularisation [91] along with altered insulin like growth factor II expression. Neither of these studies looked at sex-specific effects.

Mouse

Given the extensive use of the mouse for development studies, it is somewhat surprising that there has been little research of the effects of glucocorticoids on the mouse placenta. We have recently shown that dexamethasone exposure for 2 days around mid-gestation (day 12.5-14.5 of gestation, term=20 days) caused decreases in fetal body weight at day 14.5, but placental weight was only reduced in placentas from female fetuses [107]. These changes in placental growth were associated with sex-specific changes in placental gene and protein expression: at day 14.5, the placentas from female fetuses had higher mRNA levels of expression of *11 β HSD2* and *VEGF*, whilst protein levels of Mitogen-activated protein kinase were significantly reduced. By day 17.5, some 3, days after cessation of the dexamethasone, fetal

and placental weights are restored but levels of 11 β HSD2 protein are elevated in the placentas of female fetuses. These sex-specific changes in gene and protein levels were not present for nutrient transporters such as glucose transporter 1 and 3 or the major amino acid transports [107].

Spiny mouse

We have also utilised a precocial rodent, the spiny mouse (*Acomys cahirinus*), in which the natural circulating glucocorticoid is cortisol, not corticosterone like other rodents [110], to explore sex-specific effects of glucocorticoids on the placenta. O'Connell et al [66] examined the immediate and long-term consequences of excess maternal glucocorticoids (dexamethasone) administered for a short time (60h) at mid-gestation (day 20, term is 39 days) on placental structure and gene expression. The immediate consequences of glucocorticoid administration were similar between male and female placentas. However, two-weeks post-treatment (day 37), the transcriptional and structural response of the placenta was dependent on the sex of the fetus. Placentas of male fetuses were found to have an increase in the expression of a gene involved in placental patterning, glial cell missing 1 gene; *GCM1*, but also decreases in the expression of the primary placental glucose transporter (solute carrier family 2 (facilitated glucose transporter), member 1; *SLC2A1*). Placentas of male fetuses also had decreased amounts of maternal blood sinusoids, which are involved in the drawing of nutrient poor blood away from the placenta and back into the maternal circulation. Placentas of female fetuses were observed to have increased glucose transporter expression, and an increased amount of maternal blood sinusoids, in other words, the response of a female placenta to excess glucocorticoids was opposite to that of a male. This study highlights that while the immediate response to excess glucocorticoids may be the same for both sexes in this species, these may persist or evolve within the placenta differently, depending on the sex of the fetus [66].

7. Significance

There is now a growing body of evidence to suggest that the placenta of a male and female differs and that this may underlie the greater vulnerability of males to stressors that occur during pregnancy. Here we provided evidence that the placental response to changes in maternal glucocorticoid status differs depending on the sex of the fetus and raises the important question: are differences in fetal outcomes driven by the fetus itself or the placenta. We suggest that the placenta should become an organ of greater interest to clinical obstetrics and perinatology, particularly with respect to how the placenta may function differently for a male and female fetus during periods of high glucocorticoid exposure.

With respect to the clinical use of glucocorticoids, the different response of a male and female to even a small dose of synthetic glucocorticoids must be followed up in a large clinical based study. At least from experimental data, the question has been raised, "Should the sex of the fetus be taken into consideration when synthetic glucocorticoids are administered during pregnancy"?

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