Developmental programing: impact of testosterone on placental differentiation

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Abstract

Gestational testosterone treatment causes maternal hyperinsulinemia, intrauterine growth retardation (IUGR), low birth weight, and adult reproductive and metabolic dysfunctions. Sheep models of IUGR demonstrate placental insufficiency as an underlying cause of IUGR. Placental compromise is probably the cause of fetal growth retardation in gestational testosterone-treated sheep. This study tested whether testosterone excess compromises placental differentiation by its androgenic action and/or via altered insulin sensitivity. A comparative approach of studying gestational testosterone (aromatizable androgen) against dihydrotestosterone (non-aromatizable androgen) or testosterone plus androgen antagonist, flutamide, was used to determine whether the effects of testosterone on placental differentiation were programmed by its androgenic actions. Co-treatment of testosterone with the insulin sensitizer, rosiglitazone, was used to establish whether the effects of gestational testosterone on placentome differentiation involved compromised insulin sensitivity. Parallel cohorts of pregnant females were maintained for lambing and the birth weight of their offspring was recorded. Placental studies were conducted on days 65, 90, or 140 of gestation. Results indicated that i) gestational testosterone treatment advances placental differentiation, evident as early as day 65 of gestation, and culminates in low birth weight, ii) placental advancement is facilitated at least in part by androgenic actions of testosterone and is not a function of disrupted insulin homeostasis, and iii) placental advancement, while helping to increase placental efficiency, was insufficient to prevent IUGR and low-birth-weight female offspring. Findings from this study may be of relevance to women with polycystic ovary syndrome, whose reproductive and metabolic phenotype is captured by the gestational testosterone-treated offspring.


Introduction

The endocrine, nutritional, and metabolic environment of the fetus programs its anatomy and physiology, and these changes probably persist into postnatal life leading to adult pathologies (Barker 2004, Gluckman et al. 2008, Nijland et al. 2008, Gabory et al. 2011, Padmanabhan & Veiga-Lopez 2011). Exposure of the fetus to excess steroids in utero has been found to alter fetal developmental trajectory and induce adult reproductive and metabolic pathologies (Abbott et al. 2006, Padmanabhan & Veiga-Lopez 2011). Specifically, gestational testosterone treatment was found to induce intrauterine growth retardation (IUGR) and low-birth-weight female offspring (Manikkam et al. 2004, Steckler et al. 2005, Godfrey et al. 2011), culminating eventually in adult dysfunctions manifested at both reproductive and metabolic levels in the female (Abbott et al. 2006, Padmanabhan & Veiga-Lopez 2011). Of translational relevance, IUGR and low birth weight have been identified as risk factors for many adulthood reproductive, metabolic, and endocrine disorders (Barker 2006, Phillips et al. 2006, Simmons 2009). IUGR is also associated with a six to ten times increase in the risk of perinatal mortality in the USA (Ananth & Wilcox 2001, Gould et al. 2003).

Several sheep models of IUGR demonstrate placental insufficiency as an underlying cause of fetal growth retardation (Regnault et al. 2002, Louey et al. 2003, Morrison 2008). For instance, IUGR induced by mid-gestation hypothermia in sheep is associated with a reduced placental mass, uterine and umbilical blood flow, transplacental amino acid flux, glucose, and oxygen transport capacity (Rees et al. 1998). In many of these IUGR models, the placenta undergoes advanced differentiation to increase the efficiency in an effort to overcome fetal growth retardation (Penninga & Longo 1998, Gardner et al. 2002, Vonnahme et al. 2006). Failure to adequately compensate appears to underlie...
IUGR and low birth weight outcomes. Conceivably, a similar placental insufficiency underlies the IUGR evidenced in gestational testosterone-treated females.

As testosterone can be aromatized to estrogen, any impaired placental function in the gestational testosterone-treated model may be mediated via androgenic or estrogenic actions of testosterone. Alternatively, as gestational testosterone treatment appears to disrupt maternal insulin homeostasis (Abi Salloum et al. 2012), effects of testosterone may also involve metabolic perturbations. In support of this, histological and/or morphological changes in human placenta are evident in women with type 1 and gestational diabetes (Higgins et al. 2011, Rossi et al. 2012). This study was carried out to test the following hypotheses: i) gestational testosterone excess compromises placental differentiation, ii) placental compromise is facilitated by androgenic actions of testosterone, iii) effects of testosterone on placenta involve altered insulin sensitivity, and iv) placental compromise in gestational testosterone-treated females involves both androgenic and metabolic pathways.

Materials and methods

Animals and gestational treatments

All procedures used in this study were approved by the Institutional Animal Care and Use Committee of the University of Michigan and are consistent with the National Research Council's Guide for the Care and Use of Laboratory Animals. The study was conducted at the University of Michigan Research Facility (Ann Arbor, MI, USA; 42°18’N) using multiparous Suffolk breed of sheep. Beginning ~3 weeks before the time of breeding, ewes were group fed daily with 0.5 kg shelled corn and 1.0–1.5 kg alfalfa hay/ewe to increase energy balance. After breeding, all ewes were housed in the same pasture and group fed daily with 1.25 kg alfalfa/brome mix hay per ewe, ensuring that they meet the nutrient requirements for sheep as defined by the Committee on the Nutrient Requirements of Small Ruminants (NRC 2007). Purebred registered Suffolk sheep rams obtained from the provider were raddled on their chests and housed with the females. Mating was confirmed by the presence of paint on the backs of the females as a result of mounting. Once mated, the females were randomly assigned to the different treatment groups, blocking for body condition and weight.

The treatment groups used in this investigation include the following: a control, a gestational testosterone-treated, a gestational dihydrotestosterone (DHT)-treated, a gestational testosterone plus androgen antagonist-treated (TF), a gestational testosterone plus insulin sensitizer-treated (TR), and a gestational testosterone plus androgen antagonist and insulin sensitizer-treated (TFR) group. Details of gestational testosterone, DHT, and androgen antagonist (flutamide) treatments, husbandry, and nutrition of maternal sheep and their impact on reproductive function have been published previously (Manikkam et al. 2004, Steckler et al. 2007, Jackson et al. 2008).

In brief, for the generation of gestational testosterone- and DHT-treated groups, pregnant sheep were administered intramuscularly twice a week from days 30 to 90 of gestation with either 100 mg testosterone propionate (~1.2 mg/kg; Sigma Chemical Co.) or 100 mg DHT propionate (Steraloids, Inc., Newport, RI, USA) suspended in 2 ml cottonseed oil (Manikkam et al. 2004, Steckler et al. 2007). The generation of the TF group involved co-treatment of testosterone with an androgen antagonist, flutamide (Sigma–Aldrich; 15 mg/kg per day, oral; Jackson et al. 2008). The generation of the TR group involved co-treatment of testosterone with an insulin sensitizer, rosiglitazone (Avandia, GlaxoSmithKline; 8 mg/kg per day, oral). The generation of the TFR group involved co-treatment of testosterone with both an androgen antagonist and an insulin sensitizer.

The testosterone and DHT doses were chosen to simulate the doses used in earlier neuroendocrine and ovarian investigations of offspring from treated mothers (Manikkam et al. 2004, Steckler et al. 2005, 2007, Jackson et al. 2008, Padmanabhan & Veiga-Lopez 2011). The concentrations of testosterone achieved in maternal circulation with this mode of testosterone delivery are in the range of adult males (Veiga-Lopez et al. 2011). The concentrations of testosterone achieved in female fetuses with this mode of testosterone delivery are also within the range observed in the control fetal males (Veiga-Lopez et al. 2011). The chosen flutamide dose has been found to effectively negate the masculinizing effects of both endogenously produced and exogenously administered testosterone (Jackson et al. 2008). The rosiglitazone dose chosen has been demonstrated to be effective in restoring insulin sensitivity in adult prenatal testosterone-treated females (Veiga-Lopez et al. 2010) and normalizing the increase in insulin/glucose ratio observed in gestational testosterone-treated females (Abi Salloum et al. 2012).

Experimental design

Study 1: impact of excess testosterone on placental differentiation

To determine the effects of gestational excess of testosterone on placental differentiation, pregnant control (n=20) and testosterone-treated (n=22) sheep were anesthetized as described previously (Veiga-Lopez et al. 2011) on day 65 (mean ± S.E.M., 64.9 ± 0.1) (control, n=10 and testosterone, n=10) of gestation (term, ~147 days), and uterus and fetuses were removed. Fetal weight was recorded. After uterus removal, the cotyledonary–caruncular units (placentomes) of the placenta were dissected out from the uterine wall, and the placentome morphology was assessed as described previously (Vatnick et al. 1991). Placentomes were classified into four types (A, B, C, and D) based on their stage of development, which is determined by the relative predominance of the caruncular (maternal) compartment vs the cotyledonary (fetal) compartment (see Fig. 1 for details). During the first placentome stage (type A), the caruncular tissue is predominant and surrounds the cotyledonary tissue. As gestation progresses, the fetal tissue protrudes over the caruncular tissue (type B) until both compartments are flattened and occupy similar surface areas (type C). In the final stage of differentiation (type D), the
cotyledonary tissue occupies the majority of the surface area in view of an increased nutrient and oxygen demand at later gestational stages (Vatnick et al. 1991). For each uterus, placentome count and weight were recorded, and placental efficiency was calculated as the ratio between the total fetal weight and the total placental weight.

**Study 2: androgenic programing of placentome differentiation**

A comparative approach of studying gestational testosterone (aromatizable androgen) and DHT (non-aromatizable androgen) treatments was used to determine whether the effects of testosterone on placentome differentiation were programed by its androgenic actions. Pregnant control, testosterone-treated, and DHT-treated females were studied on either day 90 (mean ± S.E.M., 89.9 ± 0.1; n = 7, 8, and 15 respectively) or day 140 (mean ± S.E.M., 139.9 ± 0.1; n = 6, 13, and 15 respectively) of gestation, at which point fetuses and uteri were harvested. Assessment of placental gross morphology was performed as described above.

**Study 3: androgenic vs metabolic progr"amming of placentome differentiation**

As DHT (the androgen used in Study 2) can be converted to 5α-androstane,3β,17β-diol (3β-diol) and can therefore bind to estrogen receptor 2 (Handa et al. 2008), co-treatment of testosterone with the androgen antagonist was used to confirm androgenic mediation. To establish whether the effects of gestational testosterone treatment on placentome differentiation may involve metabolic compromise, the insulin sensitizer, rosiglitazone, was used for co-treatment with testosterone. Two studies were carried out. The first included control (n = 8), testosterone (n = 11), and TF (testosterone + androgen antagonist; n = 9) pregnant sheep. The second study included control (n = 12), testosterone (n = 12), TF (testosterone + androgen antagonist; n = 10), and TR (testosterone + insulin sensitizer; n = 10)-treated pregnant sheep. The first study was carried out on days 91.5 ± 0.1 (mean ± S.E.M.) of gestation and the second on day 90.5 ± 0.1 of gestation. Assessment of placental changes was performed as described above.

To test whether both androgenic and metabolic pathways are involved in placental compromise of gestational testosterone-treated sheep, control, testosterone-treated, and TFR (testosterone + androgen antagonist + insulin sensitizer)-treated pregnant females were studied on day 60 (mean ± S.E.M., 59.5 ± 0.1; n = 9, 10, and 9 respectively) of gestation. Removal of fetuses and uteri and assessment of placental gross morphology were carried out as described above.

Weights of breeder animals, maternal weight at the time of placental collection, and birth weight of offspring from parallel cohorts

Weights of breeder animals at the time of mating (except Study 1) and maternal weight at the time of placental collection (except Study 2, day 90) were recorded. For the study of androgenic programing of placentome differentiation at days 90 and 140 of gestation and combined androgenic and metabolic programing at day 60, when more animals were available, a parallel set of pregnant females was maintained for lambing. The birth weight of their offspring was recorded. The number of offspring born, treatment groups, and offspring gender distribution for each of these studies are as follows: androgenic programing at day 90 (females: control, 3; testosterone, 10; and DHT, 6; males: control, 3; testosterone, 7; and DHT, 8), day 140 (females: control, 18; testosterone, 23; and DHT, 6; males: control, 26; testosterone, 20; and DHT, 9), and combined androgenic and metabolic programing at day 60 (females: control, 7; testosterone, 3; and TFR, 11; males: control, 8; testosterone, 5; and TFR, 6).

**Statistical analysis**

Placental efficiency was calculated as a ratio of total fetal weight to placentome weight and excluded weights of both amniotic and chorioallantoic sacs. The differences in the weight and number of each type of placentome, placental efficiency, and fetal weight between treatment groups were compared after appropriate transformations to account for heterogeneity of variances. To study the effect of excess testosterone on placentome differentiation, an independent samples t-test was used to evaluate the differences in the number and weight of placentomes, placental efficiency, and fetal weight at gestational day 65.

To test the effects of androgens on placentome differentiation, the following analyses were performed. ANOVA followed by Dunnett’s post hoc test was used to compare the differences in the number and weight of placentomes, placental efficiency, and fetal weight between control and treatment groups at days 90 and 140. At gestational day 140, the same analysis was also performed separating females and male fetuses. The number of fetuses per dam was used as a covariate in the statistical model.

To test androgenic vs metabolic programing of placental differentiation, the following analyses were carried out. As two cohorts were used (see ‘Materials and methods’ section for details), and no statistical differences within treatment group were found when comparing control, testosterone, and TF groups between the two studies, both cohorts were merged for
Results

Effects of excess gestational testosterone on placentome differentiation

On day 65 of gestation (midway of the gestational treatment regimen), the total placentome number was similar between control and testosterone-treated dams (Fig. 2). The majority of placentomes were of type A with very few types B, C, and D placentomes. The number (P<0.05) of type A placentomes was significantly lower in testosterone-treated dams compared with controls. The total weight of all placentomes was significantly lower in testosterone-treated dams compared with controls (P<0.05). The total weight of type A placentomes was also lower in testosterone-treated dams (P<0.01). There were no differences in the number of fetuses per dam or fetal weight per dam. Placental efficiency was, however, significantly higher in dams treated with testosterone (P<0.01) compared with controls.

Androgenic programing of placentome differentiation

There were no differences in the total number or weight of placentomes between control, testosterone, and DHT-treated dams (Fig. 3). Similar to day 65 of gestation, type A placentomes were still predominant at day 90 of gestation (the end of the testosterone treatment period), but with an increased proportion of types B, C, and D placentomes. No significant differences in the number and weight of type A placentomes were evident between testosterone-treated and control dams at this time point. Conversely, the number (P<0.01) and weight (P<0.01) of type C placentomes were significantly higher in the dams that were treated with testosterone relative to controls. A similar increase in the number (P<0.01), but not the weight of type D placentomes, was also evident in testosterone-treated dams. Similar effects, namely an increase in the number and weight of types C and D placentomes, were evident in DHT-treated dams. In addition, a tendency for a decrease (P=0.057) in the number of type B placentomes was evident in DHT-treated, but not testosterone-treated dams. There were no differences in the number of fetuses born, total fetal weight, or placental efficiency at this time point.

A week before lambing (gestational day 140) and ~50 days after the conclusion of gestational testosterone treatment, a shift toward more advanced placentome types (B, C, and D) was evident in the control group compared with earlier gestational time points. There were no differences between control and testosterone-treated females relative to the total number and total weight, nor in the distribution of placental types (Fig. 4). The only difference noted was in the DHT-treated group. This was reflected as a decrease in the number (P<0.05) and weight (P<0.05) of type B placentomes compared with the control group. There were no differences in the total fetal number per dam, nor in the total fetal weight per dam. When female fetuses were analyzed separately, fetal weight was lower in testosterone females compared with controls, but did not reach statistical significance with the small sample size. Placental efficiency, however, was significantly lower in testosterone-treated
in type C placentomes was partially reversed by both androgen antagonist and insulin sensitizer co-treatments. The number and weight of type C placentomes were both similar to the control group in TR-treated females while the number (P<0.05), but not the weight, of type C placentomes was significantly higher in TF-treated females compared with control females.

In type D placentomes, the total number (P<0.01) and weight (P<0.01) were higher in testosterone-treated animals compared with controls. Similarly, the count and weight of type D placentomes were also significantly higher in both TF-treated (P<0.05 for both variables) and TR-treated (P=0.052 and P<0.01 respectively) dams. There were no differences in the total number and total weight of fetuses or placental efficiency among the four treatment groups.

Combined treatment with an androgen antagonist and an insulin sensitizer could not rescue the control

(P<0.05), as well as in DHT-treated (P<0.05), dams compared with controls.

Androgenic and metabolic mediation of placentome differentiation

The impact of androgenic vs metabolic mediation was studied at gestational day 90 (end of the testosterone treatment). No differences were evident in the total number and weight of placentomes across treatment groups (Fig. 5). Testosterone treatment decreased the number (P<0.05) and weight (P<0.05) of type B placentomes and increased the number (P<0.01) and weight (P<0.01) of types C and D placentomes, relative to the control group.

Androgen antagonist treatment reversed the effects of testosterone on the number and weight of type B placentomes, while insulin sensitizer co-treatment had no effect. The increase in the number and weight observed

Figure 3 (A) Total (top left) and number of types A, B, C, and D (top right) placentomes on gestational day 90 from control (open bars), gestational testosterone-treated (closed bars), and gestational DHT-treated (dashed bars) dams. Total weight (g, left) and weights of type A, B, C, and D (right) placentomes for the same set of animals are shown at the bottom. (B) Placental efficiency (top) and fetal weight (bottom). (C) Representative images of placentomes from each treatment. Data are expressed as mean ± S.E.M. Asterisks represent statistical differences between treatment groups (P<0.05). Body weights of breeder animals (mean ± S.E.M., kg) at the time of mating (control (C), 83.4 ± 2.9; testosterone (T), 80.0 ± 4.2; and DHT, 80.3 ± 3.1) did not differ significantly among groups.

Figure 4 (A) Total (top left) and number of types A, B, C, and D (top right) placentomes on gestational day 140 from control (open bars), gestational testosterone-treated (closed bars), and gestational DHT-treated (dashed bars) dams. Total weight (g, left) and weights of type A, B, C, and D (right) placentomes for the same set of animals are shown at the bottom. (B) Placental efficiency (top) and fetal weight (bottom). (C) Representative images of placentomes from each treatment. Data are expressed as mean ± S.E.M. Asterisks represent statistical differences between treatment groups (P<0.05). Body weights of breeder animals (mean ± S.E.M., kg) at the time of mating (control (C), 83.4 ± 2.9; testosterone (T), 80.0 ± 4.2; and DHT, 80.3 ± 3.1) did not differ significantly among groups.
phenotype in terms of advanced placental differentiation in testosterone-treated females (Fig. 6) on day 65 of gestation. Testosterone, as well as TFR-treated females showed similar advancement in the placental number and weight (tendency) with a larger number of types B + C + D placentomes present in both groups relative to controls.

**Impact of testosterone/DHT treatment and intervention on birth weight**

Gestational testosterone treatment produced low-birthweight female offspring in three of the cohorts where subsets of animals were maintained in parallel until delivery (corresponding data from groups of animals assigned for placental and fetal measures are shown in Figs 3, 4 and 6). DHT treatment reduced the birth weight in one set (Fig. 7A, top left), but not in the other (Fig. 7A, top middle). Co-treatment with an androgen antagonist plus an insulin sensitizer did not completely normalize the birth weight (Fig. 7A, top right panel, gray bar). Testosterone treatment reduced the birth weight of male offspring in one cohort (Fig. 7A, lower middle panel), but not the other two (Fig. 7A, lower left and right panels). The fetal weight of testosterone-, TF-, and TR-treated female and male fetuses was similar to controls at day 90 of gestation (Fig. 7B).
### Discussion

Findings from this study provide evidence that gestational testosterone treatment advances placental differentiation and that this advancement is facilitated at least in part by androgenic actions of testosterone and is not a function of disrupted insulin homeostasis. Advanced placental differentiation was sufficient to maintain placental efficiency during early stages of gestation, but not at later stages, culminating in IUGR and low birth weight mainly in the female offspring. The evidence in support of these conclusions and the implication of these findings are discussed below.

Testosterone, the gestational treatment used in this study, can induce a multitude of effects by directly signaling through the androgen receptor or via conversion to estrogen in tissues with aromatase activity, such as the placenta (France et al. 1987), thereby inducing estrogen-mediated actions. The conclusion that advanced placental differentiation is mediated in part by androgenic, and not estrogenic, action is supported by the finding that gestational treatment with the non-aromatizable androgen DHT, also advanced placental differentiation. The finding that the effects of DHT are mediated by androgenic action and not by conversion of DHT to 3β-diol, which is capable of binding and signaling through estrogen beta receptor (Handa et al. 2008), is corroborated by the fact that co-treatment with flutamide, an androgen antagonist, partially prevented the advancement in placental differentiation. Incomplete reversal may be a function of inadequate blockade with an androgen antagonist or suggestive of multiple mechanisms working in concert to advance placental differentiation. Our earlier finding that the dose of androgen antagonist used was sufficient to prevent phenotypic masculinization in male offspring of gestational testosterone-treated animals (Jackson et al. 2008) suggests that dosage of flutamide used is appropriate. Androgen receptors have been shown to be present in the human and bovine placenta (Horie et al. 1992, Uzelac et al. 2010, Khatri et al. 2013). Furthermore, it is known that androgen receptor regulates gene networks involved in cell growth and differentiation (Martyniuk & Denslow 2012), including bone and muscle growth (Otto-Duessel et al. 2012). We have previously shown that gestational testosterone excess reprograms the developmental trajectory of the insulin-like growth factor (IGF)/insulin-like growth factor binding protein (IGFBP) system in female fetuses to reduce IGF bioavailability during IUGR (Crespi et al. 2006). Considering that the IGF system also plays a critical role in placental development (Forbes & Westwood 2008, Roberts et al. 2008, Bowman et al. 2010), accelerated placental differentiation may be a function of the altered IGF system, as appears to be the case in the fetus.

As studies with primates have found that gestational testosterone treatment impairs maternal glucose tolerance (Abbott et al. 2010) and as our preliminary findings in sheep found that gestational testosterone excess induces maternal hyperinsulinemia (Abi Salloum et al. 2012), the possibility exists that the effects of excess testosterone on placental advancement may be mediated also in part via perturbation of insulin–glucose homeostasis. The fact that a complex relationship exists between androgenic stimulation and insulin signaling has been widely recognized and is evidenced in the placental tissue in both observational and interventional studies. For example, the placentae obtained from the pregnant uteri of human with gestational diabetes have increased androgenic activity with reduced aromatase protein levels and an increased abundance of androgen receptors (Uzelac et al. 2010). A direct effect of insulin on suppressing aromatase activity in the human trophoblast cells has been demonstrated (Nestler 1993). In addition, insulin sensitization indirectly affects the placenta via circulating adipokines, such as adiponectin (McDonald & Wolfe 2009), which regulates extensive gene expression networks including steroidogenic enzymes in the human placenta. However, findings from this study that the insulin sensitizer, rosiglitazone, did not alter the effects of testosterone on placental development indicates that perturbations in the insulin signaling pathway are not a major mediator behind the actions of testosterone described in this study.

The advanced differentiation of placentomes is probably a compensatory approach to provide adequate nutrition for the fetus. Visual observation indicated that the fetal compartment of the placenta appears more vascular in testosterone-treated females than controls. The finding that placental efficiency was increased.

### Figures

**Figure 7**
(A) Birth weight of female and male offspring from three of the cohorts used in the placental studies. Placental and fetal data from the subset of animals from these same breeding cohorts raised in parallel are shown in Figs 3, 4 and 6. (B) Fetal weights of day 60 fetuses segregated by sex (corresponding Fig. 5).

![Birth weight and fetal weight data](https://example.com/birth-weight-fetal-weight-data.png)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Females birth weight (kg)</th>
<th>Males birth weight (kg)</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.5</td>
<td>6.8</td>
</tr>
<tr>
<td>Testosterone</td>
<td>7.2</td>
<td>9.3</td>
</tr>
<tr>
<td>DHT</td>
<td>8.5</td>
<td>11.2</td>
</tr>
<tr>
<td>TR</td>
<td>9.8</td>
<td>12.5</td>
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![Graph showing birth weight and fetal weights](https://example.com/birth-weight-fetal-weights.png)
day 65 of gestation (Fig. 2), comparable to that of controls at day 90 of gestation (Fig. 3), and reduced at day 140 of gestation (Fig. 4) is consistent with placental advancement being able to compensate during early pregnancy, but unable to do so as pregnancy progressed. The absence of change in placental efficiency in testosterone-treated sheep compared with controls at day 90 of gestation (Fig. 3), in the face of advanced placental differentiation, suggests that this advancement is a means by which placental efficiency is maintained at this age. By contrast, later in pregnancy, when fetal growth is at its maximum, this compensatory mechanism appears to be insufficient to meet the high demands of the growing fetus. This is supported by findings of reduced placental efficiency at day 140 of gestation (Fig. 4). While no differences were evident in the total fetal weight of control and gestational testosterone-treated fetuses at day 140 (Fig. 4), female offspring of gestational testosterone-treated animals from the same cohort of animals maintained in parallel until delivery were of low birth weight. This was also the case with the other two cohorts maintained until birth (Fig. 7). In sheep, the last week of fetal growth can account for as much as 10 or 17% of the total birth weight in single and twin pregnancies respectively (Koong et al. 1975). Therefore, it is likely that the additional week of maturation time, when fetal growth is at its highest, accounts for the difference between the weights recorded at day 140 (similar weights between controls and testosterone-treated) vs at birth (lower weights in testosterone-treated females). In all these studies, consistent with our earlier findings (Manikkam et al. 2004), the birth weight of female offspring born to testosterone-treated mothers was lower compared with controls (Fig. 7).

The birth weight of males was not affected by gestational testosterone excess from days 30 to 90 of gestation, but for one cohort (Fig. 7). It is unclear as to why IUGR was only restricted to this cohort in males. Low birth weight of male offspring has been reported when testosterone treatment was extended through day 120 of gestation (Recabarren et al. 2008). One possibility for the differential effects on male birth weight among cohorts is that the impact of steroid excess at the placental level was stronger in this cohort. In support of this, among all cohorts studied, placental efficiency was most significantly compromised in the pregnancy cohort where male birth weight was also affected (day 140; Fig. 4). It is possible that the stronger effect on this cohort may have also been influenced by maternal body condition, which can affect placental function and fetal growth in sheep (Osgerby et al. 2003). Conceivably, male fetuses are more resilient to the homeostatic imbalance and placental disruptions induced by testosterone. Previous evidence supports gender-specific resilience to placental changes (Misra et al. 2009).

While the focus of this discussion is on IUGR/birth weight as it relates to placental efficiency, it needs to be recognized that developmental disruptions in organ systems may persist and manifest as adult disorders, even when advanced placentome development may be able to overcome IUGR and low birth weight. We have previously shown that female lambs born after exposure to excess testosterone at the doses used here during the critical gestational period for reproductive organ differentiation develop reproductive and metabolic dysfunctions in adulthood, exemplifying the developmental programming of adult diseases (Padmanabhan et al. 2010, Padmanabhan & Veiga-Lopez 2013). From the evolutionary perspective, it may be more crucial to maintain the normal gestational weight to ensure perinatal survival, than to protect the endocrine organs during fetal development, so compensatory mechanisms may not have evolved for the latter.

Compromised placental differentiation, IUGR, and low birth weight are probably a function of inadequate nutrient support. During normal pregnancy, type D placentomes increases during late gestation to support the increasing demand of the growing fetus (Alexander 1964a). In response to maternal undernutrition (Symonds et al. 1998, Heasman et al. 1999) or hypoxia (Penninga & Longo 1998), an increase in type D placentomes occurs early during pregnancy to increase nutrient transfer in order to compensate for the reduced nutritional intake. On the other hand, overnutrition of adolescent sheep (Wallace et al. 2000) leads to a predominance of type A placentomes in late gestation, which is accompanied by a reduction in uterine and umbilical blood flow (Wallace et al. 2000). The finding that no difference in placentome numbers was evident at any gestational age, combined with a decline in the placental weight at fetal day 65 (Fig. 2) of gestational testosterone-treated sheep, is consistent with previous findings, in that reduced placental weight appears to be the result of differences in the mean weight of placentomes rather than a reduction in placentome numbers (Alexander 1964b, Bell et al. 1987, Wallace et al. 2000).

The compromised placental differentiation (probably function) and low birth weight (Fig. 7) of gestational testosterone-treated sheep may be a function of reduced progestogenic support to the developing fetus. Our preliminary findings in sheep found that gestational testosterone treatment compromises progesterone production at day 90 of gestation (Abi Salloum et al. 2012). Progesterone is essential to support immunological adaptation to pregnancy (Szekeres-Bartho et al. 2001, Di Renzo et al. 2005) and create a protective immune environment. Studies in sheep have found that low progesterone levels during pregnancy are associated with the low birth weight (Wallace et al. 1997). The finding of reduced progestogenic support and low-birth-weight female, but not male, offspring in gestational
testosterone-treated pregnancies is similar to findings in a human cohort study (Hartwig et al. 2013). Another study in sheep found that maternal periconceptional undernutrition increased maternal progesterone with no impact on the birth weight (Debus et al. 2012). Evidence points to paternal/maternal genes influencing placental imprinting and, thus, having an impact on placental transfer of nutrients (Reik et al. 2003). While considering reduced progesterogenic support as a cause or consequence of placental insufficiency, it is important to recognize that gestational testosterone treatment could alter progesterone metabolism at the level of the fetoplacental unit (Carrizo et al. 1994, Grzesiak et al. 2014). Previous studies point to the influence of stress on reduced progesterone levels (Parker & Douglas 2010). Gestational testosterone excess may be viewed as a stressor in this regard.

Although not closely related phylogenetically, the sheep and the human placenta are of a structurally similar type. This is particularly reflected by the architecture of the fetal villous tree, with stem, intermediate, and terminal vessels. The terminal vessels, or capillary complex, which are the functional unit of exchange, differs to a lesser extent between the sheep and human. In both, chorioallantoic villous trees build up to fetal–placental cotyledons. In the sheep, one cotyledon penetrates the maternal septal system of the endometrial caruncle, both together forming one of the numerous placentomes. In humans, cotyledons protrude into the extended maternal intervillous blood space of the single polycyotyledonary placental disc (Kauffmann et al. 1985, Mossman 1987, Leiser 1991, Leiser & Kaufmann 1994, Leiser et al. 1997, Parker & Douglas 2010). As such, findings from this study may have implications relative to placental development in pregnant women with elevated androgen levels, such as polycystic ovary syndrome (PCOS; Sir-Petermann et al. 2002, 2012) or congenital adrenal hyperplasia (Lo & Grumbach 2001). It is of interest in this regard that female offspring of gestational testosterone-treated sheep manifest the reproductive and metabolic features of women with PCOS (Padmanabhan et al. 2010, Padmanabhan & Veiga-Lopez 2011, 2013). Pregnancy complications are common with PCOS pregnancies (Qin et al. 2013). In general, pregnancy complications arise from defective placenta or trophoblast invasion (Jindal et al. 2007, Longtine & Nelson 2011). Recent studies have found placental alterations (Palomba et al. 2013) and altered steroid production (Maliqueo et al. 2013) in PCOS pregnancies. Interestingly, gestational diabetic mothers also have high levels of androgens (Sir-Petermann et al. 2002, 2012). Placental morphology is also altered in diabetic pregnancies, with increased androgen levels (Higgins et al. 2011).

In summary, findings from this study support placental compromise as the mechanism responsible for the IUGR and low-birth-weight offspring in gestational testosterone-treated animals. More in-depth studies are required to delineate the underlying molecular mechanisms.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References