Maternal betamethasone administration reduces binucleate cell number and placental lactogen in sheep

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Abstract

The placenta may mediate glucocorticoid-induced fetal growth restriction. Previous studies have examined effects of fetal cortisol in sheep, which reduces placental binucleate cell (BNC) number; the source of ovine placental lactogen (oPL). The effects of maternal GC are unknown. Therefore, this study examined the effects of maternal betamethasone (BET) administration on BNC number, distribution, placental oPL protein levels, and maternal and fetal plasma oPL levels. Pregnant ewes were randomized to receive injections of saline or one (104 days of gestation; dG), two (104 and 111 dG), or three (104, 111, and 118 dG) doses of BET (0.5 mg/kg). Placental tissue was collected before, during, and after the period of BET treatment. Fetal (121–146 dG) and placental (121 dG) weights were decreased after BET when compared with controls. In controls, the mean

number of BNCs increased until 132 dG and decreased thereafter. Placental oPL protein levels peaked at 109 dG and remained stable thereafter. Maternal plasma oPL levels in controls increased across gestation; fetal plasma oPL levels decreased. BNCs were reduced by 24% to 47% after BET when compared with controls at all ages studied. Placental oPL protein levels, maternal, and fetal plasma oPL levels were also reduced after BET injections, but recovered to values that were not different to controls near term. BET disrupted the normal distribution of BNCs within the placentome. These data may suggest a placental role in growth restrictive effects of prenatal maternal BET exposure through alterations in placental output of oPL, a key metabolic hormone of pregnancy.

Journal of Endocrinology (2007) **194**, 337–347

Introduction

One of the most effective and important therapies in perinatal medicine to manage pregnant women at risk of early preterm birth is the administration of synthetic glucocorticoids. Their use however, when given repeatedly has been subject to ongoing controversy and uncertainty (Newnham *et al.* 2002). Inappropriate exposure of the developing fetus to maternal glucocorticoids has been proposed as a mechanism for fetal programming (Seckl 1997). Maternal injection of synthetic glucocorticoids results not only in improved preterm respiratory function (Liggins & Howie 1972, Jobe *et al.* 1998, Moss *et al.* 2001, 2003), but also in growth restriction, altered hypothalamus–pituitary–adrenal (HPA) function, and insulin resistance in the offspring (Moss *et al.* 2001, Bloomfield *et al.* 2003, Sloboda *et al.* 2005a,b, 2007). Reduced fetal weight and alterations in fetal HPA function may be mediated by effects on the placenta.

The placental hormone, placental lactogen, is a member of the growth hormone family and secreted in humans by the syncytiotrophoblast (Handwerger & Freemerk 2000, Lacroix *et al.* 2002b). Placental lactogen is found in both the maternal and the fetal circulation (Gluckman *et al.* 1979), and during pregnancy, placental lactogen in humans may play an important role in the regulation of maternal carbohydrate, lipid, and protein metabolism (Handwerger & Freemerk 2000). In the fetus, placental lactogen has been suggested to have a role in the regulation of fetal growth, but this effect may be indirect through alterations in the maternal metabolic environment, maternal placental nutrient transfer to the fetus or may be mediated through stimulation of insulin-like growth factor (IGF) release (Oliver *et al.* 1992, Schoknecht *et al.* 1996). Ovine placental lactogen (oPL), has a molecular mass of 22 kDa and is a nonglycosylated 198 amino-acid polypeptide (Warren *et al.* 1990). It is produced by binucleate cells (BNCs), which are formed from uninucleated cells in the fetal trophoblast.

(Kappes *et al.* 1992). BNCs account for 10–20% of the cells of fetal trophoblast (Wooding *et al.* 1993). After cell maturation and migration to the fetal–maternal–placental interface, BNCs fuse with the maternal epithelium (Lacroix *et al.* 2002a). The oPL containing granules are transferred across the fetal–maternal–placental interface and released into both the maternal and the fetal vasculature (Wooding 1992). In sheep, maternal serum oPL or cotyledonary oPL mRNA is associated with much of the variation in fetal weight during gestation (Kappes *et al.* 1992). The number of BNCs decline near term, concomitant with a fall in maternal plasma oPL concentration (Wooding 1992) and in parallel with the late gestational increase in fetal cortisol levels, and the initiation of parturition in sheep (Liggins 1976, Challis *et al.* 2000, 2005). Placenta models using surgical ligation of one of the two umbilical arteries to create placental insufficiency in sheep resulted in reduced circulating oPL hormone levels in the maternal circulation, but a concomitant increase in levels in the fetal circulation (Newnham *et al.* 1986). Direct fetal cortisol infusion during late gestation has been shown to decrease the number of BNCs in the fetal trophoblast (Ward *et al.* 2002), but there are few data available regarding the effects of maternal injections of glucocorticoids on placental BNC distribution and function. Due to its potential role in regulating fetal growth and its inverse relationship with cortisol, we hypothesized that changes in placental lactogen may underlie glucocorticoid-induced fetal growth restriction. Therefore, we investigated the effects of maternal betamethasone (BET) administration on the numbers and distribution of placental BNCs, on placental oPL protein levels, and on maternal and fetal plasma oPL levels in sheep.

Materials and Methods

Animals and tissues

All experimental procedures were approved by the Animal Experimentation Ethics Committee of the University of Western Australia and/or the Western Australian Department of Agriculture.

Prenatal treatments

Pregnant Merino ewes (*Ovis aries*) bearing singleton male fetuses were studied to eliminate potential confounding effects of fetal sex on BET response. Animals were of known gestational age (day of mating=Day 0) were allocated randomly to receive maternal injections of saline or BET. All animals were injected intramuscularly with 150 mg medroxyprogesterone acetate (Depo Provera; Upjohn, Rydalmere, Australia) at 100 dG to reduce pregnancy losses due to subsequent glucocorticoid treatment as described previously (Moss *et al.* 2001, Sloboda *et al.* 2002). Pregnant animals received injections of saline ($n=30$) or one (at 104 dG), two (at 104 and 111 dG), or three (at 104, 111, and 118 dG) doses of BET. Maternal BET (Celestone Chronodose;

Schering Plough, Baulkham Hills, Australia) injections were given intramuscularly in a dose of 0.5 mg/kg body weight; saline injections were of a comparable volume (5–6 ml).

Tissue collection

Placental tissue was collected prior to (75, 84, and 101 dG), during (109 and 116 dG), and after (121–122, 132–133, and 146–147 dG) BET administration. At 109 dG ($n=6$) ewes had received one dose of BET given at 104 dG. At 116 dG ($n=6$) ewes had received two doses of BET given at 104 and 111 dG. At 121 dG and the following time points ewes had received three doses of BET given at 104, 111, and 116 dG. Pregnant ewes were killed with a captive bolt and fetuses were delivered by Cesarean section, weighed, and killed by decapitation. The major fetal organs were removed, weighed and collected for use in other studies. Changes in organ weights were previously reported (Sloboda *et al.* 2005a). Maternal and fetal plasma samples were collected, hysterectomy was performed, and placentomes were dissected from the uterus. Tissues were either snap frozen in liquid nitrogen before storage at -80°C , or fixed in 4% paraformaldehyde (Sigma Chemical Co.) according to standard procedures for future embedding in paraffin prior to sectioning.

Immunohistochemical localization and quantification of BNCs

Immunohistochemical detection of BNCs was performed on 6 μm paraffin-embedded sections. Sagittal cross sections were taken in the middle of the placentomes. A monoclonal rabbit antibody against oPL (1:500 dilution, generously donated by Prof. A Gertler) was used (Sakal *et al.* 1997, Kann *et al.* 1999, Leibovich *et al.* 2000) with avidin–biotin–peroxidase reagents (Elite Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA) as described previously (Jacobs *et al.* 1991). Tissue sections from each of the treatment and control groups were processed simultaneously to allow direct comparison between experiments. A tissue section from 146 dG placental tissue was used as a positive control. Negative controls were as follows: i) the oPL–primary antibody was substituted either by antibody dilution buffer or nonimmune rabbit serum (1:500 dilution); ii) (Seron-Ferre *et al.* 1978) the peroxidase-labeled secondary link antibody (goat anti-rabbit immunoglobulin) was substituted with PBS (pH 7.5) wash buffer; and iii) the slide section was only incubated with PBS (pH 7.5) diluent before the addition of the substrate–chromogen solution.

Image analysis

Semi quantitative analyses were performed using computerized image analysis (ImagePro Plus 4.5, Media Cybernetics, Silver Spring, MD, USA). To reduce the number of false positive counts, BNCs were counted only if >30% of the cytoplasm of a BNC was visible and at least 80% of the randomly selected field of view was covered with placental tissue (Ward *et al.* 2002). BNC localization and distribution

was analyzed within three levels, which refer to previously reported zones in a placentome (Burton *et al.* 1976). L1 contained 1, 6, 7, and 8 fields of interest and was taken in the zona intima, closely to the capsule of the placentome. L2 contained 2, 5, 8, and 11 fields of interest and refers best to the intermediate zone of the placentome. L3 contained 3, 4, 9, and 10 fields of interest and refers best to the hemophagus zone, closely taken to the chorion-allantois (Fig. 1). A total of 12 random fields of view (area $750 \mu\text{m}^2$) were counted per section of immunostained tissue at a magnification of $20\times$. Two animals for each gestational age and study group were randomly selected. At least three placentomes per animal, regardless of placentome subtype, were immunostained and at least four sections of immunostained tissue were counted per placentome. There were two study groups and eight gestational age time points, containing a total of 78 animals with over 3700 fields of interest counted.

Quantification of placental oPL protein levels: western blotting

Frozen placentome samples ($n=140$ from 78 sheep) were homogenized on ice for 1 min in radioimmuno precipitation assay (RIPA) lysis buffer (50 mM Tris-HCl (pH 7.5 Sigma Chemical Co.), 150 mM NaCl (EMD, Gibbstown, NJ, USA), 1% w/v sodium deoxycholate (Sigma Chemical Co.); 0.1% SDS (Bio-Rad), 100 mM sodium orthovanadate (Sigma Chemical Co.), 1% (vol/vol) Triton X-100 (Fisher Scientific, Ottawa, Canada), and Complete MiniEDTA-free protease inhibitors (Roche Molecular Biochemicals). Homogenates were centrifuged at 4°C at $15\,000\text{ g}$ for 15 min and supernatants were collected. Protein concentrations were determined using the Bradford assay (Bradford 1976). Proteins

were separated by electrophoresis on 12% polyacrylamide gels at 80 V at 4°C . Samples from control and treatment groups, from each gestational age, were run together on one gel to allow comparisons between groups. Separate runs were done with randomly picked controls or treated placentomes to allow comparisons across gestation. Each blot was repeated at least three times. Separated proteins were transferred onto nitrocellulose membranes (Bio-Rad Laboratories). The resultant blots were stained with S-Ponceau (0.1% w/v Ponceau S in 5% acetic acid v/v; Sigma Chemical Co.) to verify equal loading and transfer. The blots were washed with PBS and 0.1% Tween-20 (PBS-T; Sigma Chemical Co.) and incubated for at least 2 h at 4°C in blocking solution (5% skim milk powder w/v in PBS) on a mechanical shaker to block nonspecific binding. Membranes were incubated with the same primary monoclonal rabbit antibody against oPL as used for immunohistochemistry, but at a dilution in the ratio of 1:1000 with blocking solution (5% skim milk powder in PBS) for 1 h. All blots were then rinsed six times for 5 min each with PBS-T and incubated with secondary antibodies conjugated to horseradish peroxidase (anti-rabbit IgG-horseradish peroxidase; Amersham Life Sciences) dilution in the ratio of 1:1000 in blocking solution (5% skim milk powder in PBS) for 1 h. Blots were washed (5 min $\times$ 6) and the antibody-antigen complex was detected using a chemiluminescence detection system enhanced chemiluminescence (ECL), Perkin-Elmer, Waltham, MA, USA). Membranes were incubated without primary antibody for negative controls. Blots were exposed to autoradiographic film (Eastman Kodak X-Omat) for visualization. oPL was identified as two close bands of 22 kDa (Fig. 6). All blots were reincubated with anti β -actin (Sigma Chemical Co.) as an internal control to allow for corrections in

Figure 1 Macroscopic view of a hamatoxylin stained placentome. A total of 12 random fields of view, grouped into three levels were counted per section of immunostained tissue.

gel loading and transfer. Band density for both the protein of interest and β -actin was quantified by densitometry using Scion Image Analysis software Alpha 4.0.3.2 (Scion, Maryland, USA). The results were expressed as the ratio of protein to β -actin as relative optical density (ROD) units.

Quantification of oPL plasma levels: RIA

Plasma oPL concentrations were measured using equilibrium RIA previously described and validated in sheep (Oliver *et al.* 1992). The minimal detectable dose was 0.2 ng/ml, the intraassay coefficient of variation was 4–6%, and the interassay coefficient of variation was 7.92%. Values are expressed in terms of recombinant oPL (M3RD86, GenTech; Arcade, JY, USA).

Statistical analysis

Statistical analysis was performed using SigmaStat Statistical Software (SigmaStat v2.03; SYSTAT Software Inc., Chicago, IL, USA). Data were first analyzed for normality and equal variance (Kolmogorov–Smirnov test). Data that were not normally distributed were log transformed to achieve normality. In control placentomes, BNC numbers, oPL protein levels, and plasma oPL hormone levels were analyzed across gestation (75–146 dG) using a one-way ANOVA (Tukey), where gestational age was the factor. In treatment groups (109, 116, 121, 132, and 146 dG) BNC numbers, placental oPL protein levels and plasma oPL levels were analyzed using a two-way ANOVA (Tukey), where gestational age and treatment were factors. The number of BNCs and the distribution (total numbers and levels L1–L3) were analyzed separately for each gestational age (109, 116, 121, 132, and 146 dG) using a two-way ANOVA (Tukey), where level and treatment were the factors. The relationship between the number of BNCs, placental oPL protein levels or plasma oPL levels with fetal and placental weights was assessed by linear regression, desired power for the performed test was accepted for values α 0.800. Data are presented as mean $\pm$ S.E.M. In all cases, statistical significance was accepted for values $P < 0.05$.

Results

Fetal and placental weight

Fetal weight increased across gestational age in control ($P < 0.001$) and BET-treated animals ($P < 0.001$; Fig. 2). After maternal BET treatment at 104, 111, and 118 dG, fetal weight was significantly reduced at 121, 132, and 146 dG when compared with control animals ($P < 0.05$). There was a significant decrease in total placental weight in control animals between 75 and 146 dG ($P < 0.05$). Total placental weight was decreased at 121 dG when compared with controls after BET administration ($P < 0.05$; Fig. 2).

Figure 2 Fetal weight and placental weight across gestation. Fetal weights are represented by circles (black, control; open, BET). Placenta weights are represented by squares (black, control; open, BET). (Significance level set as $P < 0.05$; * $P < 0.05$.)

Binucleate cells

The effect of BET on BNC number To investigate the effect of BET on BNCs numbers and distribution, immunohistochemistry was used to stain the BNCs with anti-oPL antibody. BNCs were identified as oPL positive cells within tissue sections as oPL localized predominately to the BNCs, with little immunostaining in the fetal syncytium (Fig. 3; Handwerger *et al.* 1977, Kappes *et al.* 1992, Ward *et al.* 2002). In control animals, the overall mean number of BNCs increased across gestation from 75 to 109 dG ($P < 0.05$) with peak BNC numbers at 132 dG ($P < 0.05$; Fig. 4). From 132 to 146 dG, the BNC numbers in controls decreased to values similar to those observed at 109 and 116 dG ($P < 0.05$). BET administration resulted in a significant reduction in BNCs between 109 and 146 dG to values that were 24–47% lower than in control animals ($P < 0.001$; Fig. 4). The number of BNCs at 132 dG was significantly greater than at earlier time points in pregnancy, or in BET-treated animals at term ($P < 0.05$; Fig. 4).

The effect of BET on BNC localization and distribution

The localization and distribution of BNCs in placentomes is shown in Fig. 5. In controls, the distribution of BNCs at 109 was similar between the three levels. At later time points (121 and 132 dG), BNCs were more numerous in L2 when compared with L1/L3 ($P < 0.05$). Near term the mean number of BNCs in controls were highest in L1 when compared with L3 ($P < 0.001$). A decrease in BNC number after BET exposure was observed within all three levels at each gestational age studied. At 109, 116, 121, and 146 dG, the number of BNCs at L3 was significantly lower than at L1/L2 ($P < 0.05$; Fig. 5). The coefficient of variance (CV) per animal for the whole placental (fields 1–12) was 19.21% at 75–101 dG, 12.11%–control versus 23.93%–BET at 109–121 dG ($P < 0.05$), and 14.88%–control versus 12.85%–BET at 132–146 dG. The significant differences in CV between controls and BET regarding the levels studied at 109–121 dG

Figure 3 Representative photographs of binucleate cell staining in placentomes at 116 dG in control (left panels) and betamethasone (right panels) animals. Sections were taken at L1, L2, and L3 of the placentome (Fig. 1). Negative control (insert). T, trophoblast; MS, maternal syncytium; BNC, binucleate cell. (Scale bar represents 2.5 μ m.)

(control: L1 = 7.54%*, L2 = 8.53%*, and L3 = 11.83%; and BET: L1 = 13.39%*, L2 = 15.36%*, and L3 = 20.97%; * $P < 0.05$).

Placental oPL protein levels

Placental oPL protein was identified as two close bands of 22 kDa with no other background signal (Fig. 6). In control animals, the overall oPL protein levels increased from 75 to 109 dG ($P < 0.05$), followed by a decrease from 109 to 116 dG ($P < 0.05$; Fig. 7). There was no significant further change in protein levels from 116 to 146 dG. In BET-treated animals, placental oPL protein levels were 52–72% lower than in control animals at 116 and 121 dG ($P < 0.05$; Fig. 7). There were no

significant differences in placental oPL protein levels between control and BET groups thereafter (121–146 dG; Fig. 7).

Maternal and fetal circulating oPL plasma levels

Maternal plasma oPL levels in controls significantly increased across gestation ($P < 0.05$; Fig. 8). Fetal plasma oPL levels in controls significantly decreased near term (146 dG versus 101 and 75 dG, $P < 0.05$; Fig. 8). Maternal and fetal plasma oPL levels were significantly decreased when compared with controls following BET injections (109, 116, and 121 dG, $P < 0.05$; Fig. 8). Near term, maternal, and fetal plasma oPL levels in BET recovered to values that were not significantly different from controls (Fig. 8).

Figure 4 Mean number of binucleate cells in sheep placentomes during pregnancy and after BET. Grey bars are control placentomes, white coarse bars are BET placentomes. Significant differences in BNC numbers between control and BET placentomes are represented by stars. Different letters on the histogram represent significant differences in control groups over gestation. Different numbers on the histogram represent significant differences in BET groups over gestation. (n, number of animals studied; significance level set as $P<0.05$; * $P<0.001$.)

The relationship between BNC number, oPL protein level, plasma oPL levels with fetal weight and placental weight

The number of BNCs was positively associated with fetal weight in controls, but not in BET-treated animals (Table 1). There was no significant association between BNCs and oPL protein levels, placenta weight or fetal

circulating plasma oPL levels either in controls or in BETs. Maternal circulating oPL levels showed a positive association with the number of BNCs in BETs, which was not seen in the controls.

In control and BET-treated animals, oPL protein levels were positively associated with maternal circulating oPL plasma levels (75–146 dG) and fetal weight (75–109 dG;

Figure 5 Distribution of mean number of BNCs in different levels of the placentome. Grey bars are control, black dots are BET placentomes. Black bars and white dots represent total number of BNCs (T). At each gestational age two randomly selected animals were studied. Different letters on the histogram represent significant differences in control groups at each gestational age. Different numbers on the histogram represent significant differences in BET groups at each gestational age. (Significance level set as $P<0.05$; *control versus BET in L1 to L3 $P<0.001$; **control versus BET with $P<0.001$.)

Figure 6 Representative western blots of ovine placental lactogen (a, ~22 kDa) and β -actin (b, ~42 kDa) across gestation (dG) in control- and betamethasone (B)-treated animals.

Table 1). After BET treatment there was an additional positive association between oPL protein levels and fetal circulating plasma oPL levels and placenta weight (Table 1).

In control sheep, fetal weight was inversely associated with placental weight early to mid-gestation (75–146 dG) and positively associated with late in gestation (109–146 dG). There was no association between placental weight and maternal circulating plasma oPL level, but fetal circulating plasma oPL level was positively associated with controls and BETs (Table 1). Fetal weight presented a strong association to maternal circulating plasma oPL levels across gestation in either controls or BETs (Table 1).

Discussion

Antenatal glucocorticoid administration has beneficial clinical effects for preterm infants. There are, however, a number of known deleterious effects with repeated prenatal glucocorticoid exposure. This study, to our knowledge, is the first study to show

Figure 7 Ovine placental lactogen protein levels in sheep placentomes during pregnancy and after BET. Grey bars are control, white coarse bars are BET placentomes. Significant differences in oPL protein levels between control and BET placentomes are represented by stars. Different letters on the histogram represent significant differences in control groups. Different numbers on the histogram represent significant differences between groups. (n, numbers of placentomes analyzed; significance level set as $P < 0.05$; * $P < 0.05$.)

Figure 8 Maternal and fetal plasma oPL hormone level across gestation. Maternal plasma hormone levels are represented by squares (black, control; open, BET). Fetal plasma hormone levels are represented by circles (black, control; open, BET). (Significance level set as $P < 0.05$; * $P < 0.05$.)

that repeated maternal i.m. injections of BET in the last third of gestation reduced placental BNC numbers, decreased oPL protein levels, and decreased circulating oPL levels in the mother and the fetus. We have demonstrated that BNC number, placental oPL protein levels, and circulating maternal and fetal oPL levels were positively associated with fetal weight. Furthermore, we have shown that maternal BET administration changed the distribution and localization of BNCs within the placentomes. Based on previous observations, we suggest that the demonstrated growth restricting effects of maternal BET (Johnson *et al.* 1981, Jobe *et al.* 1998, Sloboda *et al.* 2000) may be associated with our observed alterations in BNC formation and placental production of oPL.

In contrast to the human placenta, ruminant (such as sheep) placentae are synepitheliochorial and demonstrate noninvasive placentation (Handwerger *et al.* 1975). Although there are differences in morphology and in the ultrastructure of the placenta between humans and sheep, studies comparing placental vasculature between sheep and humans report that the sheep placenta in many aspects is workable as a model for the human placenta (Leiser *et al.* 1997). Placental lactogen is found in humans and sheep, but produced by somewhat different trophoblast types. Early studies in human pregnancies did not demonstrate significant changes in maternal human placental lactogen levels after glucocorticoid treatment (Maltau *et al.* 1979, Socias *et al.* 1979), others observed a significant reduction in maternal human placental lactogen after maternal dexamethasone treatment (Lange & Anthonisen 1980).

With increasing gestational age changes in placenta gross morphology and ultrastructure occur as adaptations in response to nutritional and endocrine challenges (Sibley *et al.* 2005). oPL is somatogenic; stimulating ornithine decarboxylase activity, amino acid uptake, glycogen synthesis, and IGF secretion (Anthony *et al.* 1995). During pregnancy,

Table 1 The relationship (expressed as *r*-values) between binucleate cell (BNC) number, ovine placental lactogen (oPL) protein level, plasma oPL levels with fetal weight and placental weight

		No. of BNCs	oPL protein	Fetal plasma oPL	Maternal plasma oPL	Fetal weight
oPL protein						
75–146 dG	Control	−0.300		+0.253	0.000166	−0.192
	BET	+0.519		+0.683*	+0.640*	+0.329
75–109 dG	Control	−0.063		−0.281	+0.448 ^a	+0.564*
109–46 dG	Control	+0.281		−0.137	−0.163	−0.331
Days 109 and 116	Control	+0.165		+0.402	0.0653	−0.653 ^a
	BET	−0.0917		+0.927*	+0.552	−0.535
Days 116 and 121	Control	+0.102		+0.522	+0.461	+0.448
	BET	+0.771		+0.197	0.0305	+0.268
Fetal plasma oPL						
75–146 dG	Control	−0.271	+0.253		0.0428	−0.432*
	BET	+0.240	+0.683*		+0.568*	+0.303
75–109 dG	Control	0.0253	−0.281		+0.427	−0.370
109–146 dG	Control	−0.130	−0.137		+0.219	0.0217
Maternal plasma oPL						
75–146 dG	Control	+0.507 ^a	0.00016	0.0428		+0.849*
	BET	+0.610*	+0.640*	+0.568*		+0.838*
75–109 dG	Control	+0.353	+0.448*	+0.427		+0.699*
109–146 dG	Control	+0.112	−0.163	+0.219		+0.678*
Fetal weight						
75–146 dG	Control	+0.775*	−0.192	−0.432*	+0.838*	
	BET	+0.464	+0.329	+0.303	+0.838*	
75–109 dG	Control:	+0.730*	+0.564*	−0.370	+0.699*	
109–146 dG	Control	−0.0190	−0.331	0.0217	+0.678*	
Days 116–146	Control	+0.0748	−0.248	+0.132	+0.838*	
	BET	+0.545	+0.835*	+0.660 ^a	+0.919*	
Placental weight						
75–146 dG	Control	−0.213	+0.0518	+0.502*	0.0299	−0.298*
	BET	+0.560 ^a	+0.597*	+0.582*	+0.542 ^a	+0.326 ^a
75–109 dG	Control	−0.182	−0.337	+0.372	−0.235	+0.225
109–146 dG	Control	−0.299	+0.125	+0.144	+0.291	+0.397*

Linear regression between control- and betamethasone-treated animals. Significance level set as $P<0.05$; * $P<0.05$.
^aThe power of the performed test is below the desired power of 0.800.

oPL stimulates development of the mammary glands (Ward *et al.* 2002) and redirects substrate utilization to make glucose available for transport to the fetus (Breier *et al.* 1994). oPL is produced by the BNCs and is found in both the maternal and the fetal circulation (Gluckman *et al.* 1979). It is well established that maternal stress (changes in nutrition or glucocorticoid administration) during pregnancy can restrict fetal growth and induce adult disease. Changes in gross morphology and ultrastructure of the placenta are interrelated and lead to alterations in surface area, vascularity, barrier thickness, and cell composition of the placenta, all of which influence placental transport characteristics (Sibley *et al.* 1997). It is therefore likely that altered placental morphology, growth and/or function underpin the changes in fetal growth and increased disease risk in models of prenatal under-nutrition, and/or prenatal glucocorticoid exposure. We have demonstrated that the number of BNCs in control animals increased from early (75 dG) to late (132 dG) gestation and subsequently declined to term (146 dG). The late gestational BNC decrease is most likely associated with rising fetal circulating cortisol levels at this time (Wooding *et al.* 1993, Fowden *et al.* 1998, Ward *et al.* 2002). It has been reported

previously that cortisol infusion, directly into the fetus during late gestation decreased BNC number in fetal trophoecto-derm (Ward *et al.* 2002). Further, when the natural cortisol surge at the end of gestation was abolished by fetal adrenalectomy or fetal hypophysectomy, the normal pre-partum decline in BNC numbers was not observed (Ward *et al.* 2002). We now demonstrate in the present study that administration of synthetic glucocorticoids also reduces placental BNC numbers. Previous studies suggest that chorionic BNCs deliver their products to the maternal and fetal circulation by migrating to and fusing with the fetomaternal syncytium (Wooding *et al.* 1992). Intrafetal cortisol injections have been shown to reduce BNC numbers by either increasing the rate of BNC migration across the fetal–maternal interface or inhibition of BNC formation (Ward *et al.* 2002). In our study, BET administration may have prevented the natural occurring increase in BNC numbers observed from early (75 dG) to mid-gestation (109 dG) in controls and may be indicative of inhibited BNC formation. We have shown for the first time, that BNCs are differentially distributed throughout different areas of an ovine placentomes and that BET administration significantly

altered this distribution. This has clear implication for placental function. Placenta nutrient transport has long been known to be dependent on vascular development. Placental vascularity in sheep during pregnancy occurs through an increase in the number and surface density of capillaries, particularly on the fetal side of the placentome (Stegeman 1974, Reynolds *et al.* 2005). Placental surface area for nutrient exchange increases through elongation and increased branching of the fetal villi (Stegeman 1974, Macdonald & Fowden 1997). In sheep, BNCs from the fetal chorionic epithelium migrate through the apical tight junction of the fetal trophoctoderm and fuse into the syncytium (Wooding *et al.* 1981). The observation of BNCs at all levels of the placentome suggests that the BNC population is continually developing in association with the expanding villous structure. The changes in BNC numbers with advancing gestation in L1 might be related to terminal villi development in the placentome, although this would not completely explain the higher values in L2 when compared with L1 at 121 and 132 dG. Furthermore, our observed BET induced reduction in BNCs in the superior portions of the placentomes (L2 and L3) at time points immediately following BET injections (109, 116, and 121 dG) suggests that the terminal villi development with new BNCs in L1 could explain why there are fewer numbers of BNCs in BET-treated animals in L3 when compared with L1. It also appears that placenta has the capacity to recover, given that this effect diminished later in gestation (132 and 146dG). Others have speculated that the reduction in BNCs caused by cortisol exposure influences the expansion of the fetomaternal syncytium, barrier thickness, and placental hormone secretion (Ward *et al.* 2002, Fowden *et al.* 2006). Consistent with this, placental growth impairment through undernutrition in guinea pigs resulted in a reduction surface area by 60–70% and an increase in barrier thickness between maternal and fetal capillaries by 40% late in gestation (Roberts *et al.* 2001). We speculate that maternal administration of BET might influence maternal–fetal nutrient transfer by impairment of placental surface area and barrier thickness, and have downstream effects on fetal growth. It is clear that a full understanding of this process using accurate stereological and functional investigations needs to be done.

Previous studies suggest that oPL may play an important role in fetal growth through its actions on maternal metabolism regulating fetal substrate availability (Handwerger *et al.* 1975). Although some previous studies reported higher values for maternal and fetal plasma oPL, Bauer *et al.* (1995) reported similar values to ours. The fact that maternal plasma oPL levels significantly increased with advancing gestation in control animals is not dissimilar from previous findings (Chan *et al.* 1978). oPL is detectable in maternal serum as early as 50 dG (Handwerger *et al.* 1977). Near term, the decline in BNC number is coincident with a decline of maternal serum oPL concentrations (Handwerger *et al.* 1977, Taylor *et al.* 1980, Wooding *et al.* 1993). Our data on placental oPL protein levels permit us to relate the effects of BET on BNC number to their

function. We have shown that BET exposure significantly decreased the number of BNCs from 109 to 146 dG and decreased overall placental oPL protein levels and fetal and maternal plasma oPL levels. At 132–146 dG placental oPL protein levels and fetal and maternal oPL plasma levels returned back to values that were not different from controls even though BNC number remained low. This is suggestive of fewer BNCs producing more oPL and we speculate that this increased oPL protein output may reflect a ‘functional adaptation’ of BNCs in BET exposed placentae. The exact mechanism by which glucocorticoids act on BNCs and regulate the placental lactogen secretion requires further investigation. The variation in fetal weight has been attributed to placental weight, maternal serum oPL, and cotyledonary oPL mRNA concentrations (Kappes *et al.* 1992, Fowden *et al.* 2006). In the present study fetal weight was positively correlated with maternal oPL levels and after maternal BET administration, maternal oPL levels decreased. Placental weight was not associated with maternal oPL plasma levels, but showed a positive association with fetal plasma oPL levels (75–146 dG) and with fetal weight (109–146 dG). The lack of association between fetal weight and fetal plasma oPL suggests that BET has potentially disrupted the normal relationship between the maternal–fetal–placental unit at mid-gestation and we can speculate that the growth restricting effects of BET exposure could be associated with altered substrate supply to the fetus (Handwerger *et al.* 1975).

The exact mechanisms by which glucocorticoids influence placental and fetal growth remain unclear and requires further investigation. However, our current data provide some understanding into the potential role of the placenta. Previous studies suggest a decrease in placental transport capacity through an increase in the barrier thickness between fetal and maternal capillaries, and a decrease in placental surface area (Fowden *et al.* 2006). Our data may suggest a role of prenatal BET exposure in inducing changes in placental ultrastructure. Furthermore the growth restricting effects of synthetic glucocorticoid exposure could be mediated by direct effects of placental lactogens on maternal and fetal growth factor secretion and/or function. Studies in rats have suggested that the link between placental lactogen and growth could be achieved via IGFs (Karabulut *et al.* 2001). Glucocorticoids have been shown to increase maternal serum IGF-I levels and a single course of antenatal BET resulted in a decrease of fetal serum IGF-II levels (Ahmad *et al.* 2006). A thorough understanding of how placental lactogens and other placental hormones influence fetal growth and fetal development will provide a better understanding about the role of the placenta in the fetal adaptive response.

Acknowledgements

We are grateful to Prof. A Gertler, Protein Laboratories Rehovot Ltd,) and Yissum Research Development Company of the Hebrew University of Jerusalem, for providing us with the rabbit anti-oPL antibody. We thank Adrian Jonker for his

assistance with tissue collection and Andrzej Surus for performing the oPL RIA. This study was supported by The Raine Medical Research Foundation of Western Australia (grant # 74301003), the NHMRC, the CIHR, and the Child Health Research Foundation of WA, Inc. Alana Mason did the majority of the placental tissue collection for this project. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 11 May 2007

Accepted 7 June 2007

Made available online as an Accepted Preprint

8 June 2007