

Fetoplacental growth and vascular development in overnourished adolescent sheep at day 50, 90 and 130 of gestation

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Abstract

To establish the basis for altered placental development and function previously observed at late gestation, fetoplacental growth and placental vascular development were measured at three stages of gestation in a nutritional paradigm of compromised pregnancy. Singleton pregnancies to a single sire were established and thereafter adolescent ewes were offered an optimal control (C) or a high (H) dietary intake. At day 50, the H group had elevated maternal insulin and amniotic glucose, whereas mass of the fetus and placenta were unaltered. At day 90, the H group exhibited elevated maternal insulin, IGF1 and glucose; fetal weight and glucose concentrations in H were increased relative to C, but placental weight was independent of nutrition. By day 130, total placentome weight in the H group was reduced by 46% and was associated with lower fetal glucose and a 20% reduction in fetal weight. As pregnancy progressed from day 50 to 130, the parameters of vascular development in the maternal and fetal components of the placenta increased. In the fetal cotyledon, high dietary intakes were associated with impaired vascular development at day 50 and an increase in capillary number at day 90. At day 130, all vascular indices were independent of nutrition. Thus, high dietary intakes to promote rapid maternal growth influence capillary development in the fetal portion of the placenta during early to mid-pregnancy and may underlie the subsequent reduction in placental mass and hence fetal nutrient supply observed during the final third of gestation.

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Introduction

Pregnancy in adolescent women is characterized by an increased risk of miscarriage, prenatal growth restriction, and preterm birth (Scholl *et al.* 1994, Olausson *et al.* 1999; http://www.marchofdimes.com/professionals/14332_1159.asp). These negative pregnancy outcomes are most prevalent in very young girls who have yet to complete their own body growth (reviewed in Wallace *et al.* 2006a). Indeed, data from the Camden Adolescent Pregnancy and Nutrition Project (Scholl *et al.* 1997) suggests that continued maternal growth occurs in ~50% of adolescents and, in spite of larger pregnancy weight gains and increased fat stores, is associated with a modest but significant reduction in birth weight compared with non-growing adolescent mothers. This effect is attributed to a competition for nutrients between the maternal body and her gravid uterus, which is unique to the adolescent growth period and has been replicated in our ovine model.

Thus, we have repeatedly demonstrated that overnourishing the singleton-bearing adolescent ewe

promotes rapid maternal growth at the expense of the gravid uterus. This results in the premature delivery of low birth weight lambs following spontaneous delivery close to term (Wallace *et al.* 1996, 2001, 2004a). Inadequate placental development is central to these negative outcomes and by late gestation placental mass is typically reduced by 30–40% and is associated with a corresponding reduction in both uterine and umbilical blood flows (Wallace *et al.* 2002a). This directly impacts transplacental transport of nutrients to the fetus, and hence absolute umbilical uptakes of glucose, oxygen and amino acids are attenuated, leading to a slowing of fetal growth in the final third of gestation (Wallace *et al.* 2002a, 2002b, 2003b). We originally hypothesized that these late pregnancy events are preceded and mediated by alterations in placental angiogenesis and vascular development, and, indeed, we demonstrated attenuated expression of mRNA for a number of angiogenic factors and their receptors in whole placentomes collected at mid-gestation from overnourished dams (Redmer *et al.* 2005). This is not surprising, as placental angiogenesis is

critical to the exponential increase in uterine blood flows seen throughout pregnancy (Reynolds & Redmer 1995) and is variously altered in each of the several models of compromised pregnancy in sheep (Reynolds *et al.* 2006). However, an important limitation of most of the previous studies is that changes in utero-placental growth, vascular development, and function were evaluated only at a single stage of pregnancy, usually near term (Reynolds *et al.* 2006).

The aim of this study was to utilize placental perfusion techniques and image analyses to quantify placental vascular development in both the maternal and fetal components of the placenta in putatively growth-restricted compared with normally developing pregnancies at three physiologically significant stages of gestation. In the sheep, these stages are known to coincide with rapid placental cellular proliferation (day 50; Ehrhardt & Bell 1995), exponential vascular development of the fetal cotyledon (day 90; Reynolds *et al.* 2005) and maximized placental vascularization, blood flow, and nutrient transfer functions (day 130; Bell *et al.* 1987, Reynolds *et al.* 2005). Furthermore, maternal and fetal organ growth, endocrinology, and metabolic status were determined at the three stages of gestation studied and related to the measured placental parameters.

Results

Maternal body weight and body condition changes

Weekly changes in maternal body weight and body condition are presented in Fig. 1. By approximately day 35 of gestation, maternal body weight and body condition score were elevated ($P < 0.001$) in the H versus the C groups and the relative difference between

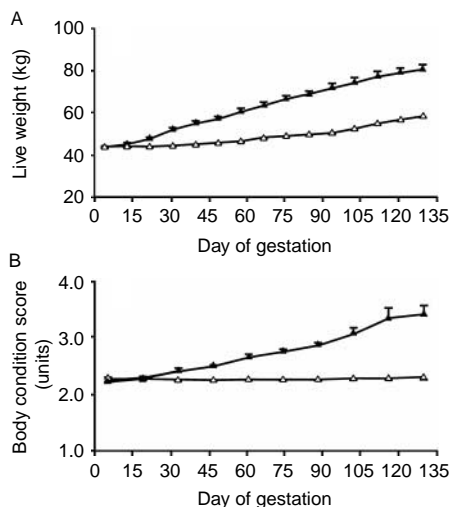


Figure 1 Weekly maternal body weight (A) and body condition score (B) changes throughout gestation in control (Δ) and high (\blacktriangle) intake adolescent dams (B). Values are means \pm S.E.M.

these groups increased as gestation advanced. Maternal body condition was maintained from embryo transfer to necropsy in all ewes in the C group, as per the experimental design.

Maternal and fetal metabolic status at necropsy

Maternal and fetal growth and metabolic status at necropsy is presented in Table 1. Compared with the C group, maternal body weight, which is the combined weight of the maternal carcass, blood, internal organs, empty alimentary tract and fat depots, was greater in the H-intake adolescent dams at all stages. Furthermore, H dietary intakes significantly elevated absolute perirenal fat at all necropsy stages compared with the C group, and increased liver weight regardless of stage of gestation. Although maternal pancreas weight was lower in C dams at the early necropsy stage (day 50), it was similar to H dams at days 90 and 130.

Concentrations of key anabolic hormones were elevated in H dams. In particular, maternal insulin concentrations were substantially greater across all stages of gestation compared with the C group (Table 1). Likewise, maternal plasma IGF1 concentrations were greater in H than C across all stages of gestation and generally increased across stage, whereas plasma glucose concentrations were significantly greater in H than C but were similar across stage. By contrast, maternal progesterone concentrations in the rapidly growing (H) dams were reduced ($P < 0.001$) during the final two-thirds of gestation.

As shown in Table 1, at day 90 of gestation, fetal weight was greater in H versus C groups, but as gestation advanced to day 130, fetal growth in the H group slowed substantially and fetal weight was reduced significantly, being 20% less than the C group. The increased fetal weight in the H versus C group at day 90 of gestation was associated with an increase in fetal brain weight; however, at day 130 of gestation absolute perirenal fat, brain, pancreas, and liver weights were not significantly different between groups. Although the stage \times nutrition interaction was weak ($P < 0.07$), at day 130 the brain:fetal weight ratio was significantly greater in the growth-restricted fetuses of the H dams ($P < 0.008$) relative to normally growing C fetuses, commensurate with fetal brain-sparing.

The observed differences in fetal weight mirrored the differences in amniotic and/or fetal plasma glucose concentrations. At day 50 of gestation, amniotic glucose concentrations were elevated in the H versus the C group. As gestation advanced to day 90, amniotic glucose concentrations increased further and paralleled the greater fetal plasma glucose concentrations in the H group at this stage compared with the C group. By contrast, in late gestation (day 130) fetal growth restriction in the H versus the C group was associated with a reduction in amniotic and fetal plasma glucose

Table 1 Maternal and fetal metabolic status and key organ weights in control (C) and high (H) intake pregnancies at necropsy on day 50, 90 or 130 of gestation.

	Day 50 of gestation			Day 90 of gestation			Day 130 of gestation			P value	Stage	Interaction
	C		H	C		H	C		H			
	N	Mean	S.E.M.	N	Mean	S.E.M.	N	Mean	S.E.M.			
Maternal measurements												
Maternal body weight (kg)	38 ± 1	48 ± 1*		37 ± 1	59 ± 2*		40 ± 1	67 ± 2* ^b		<0.001	–	
Peri-renal fat weight (g)	773 ± 128	1281 ± 77*		900 ± 57	1736 ± 158*		751 ± 83	1915 ± 156*		<0.001	<0.001	
Liver weight (g)	573 ± 16	1029 ± 50		593 ± 11	1125 ± 62		721 ± 11	1232 ± 58		<0.001	<0.001	
Pancreas weight (g)	53 ± 3	65 ± 3*		52 ± 2	61 ± 5		67 ± 5	57 ± 2 ^b		<0.001	<0.001	
Plasma insulin (IU/ml)	22 ± 2	142 ± 49		22 ± 2	116 ± 31		68 ± 18	149 ± 36 ^a		<0.001	<0.001	
Plasma IGF1 (pmol/ml)	32 ± 2	39 ± 3		50 ± 3	65 ± 2		65 ± 5	88 ± 4		<0.001	<0.001	
Plasma glucose (μmol/ml)	3.5 ± 0.2	4.1 ± 0.2		3.2 ± 0.1	4.0 ± 0.2		3.3 ± 0.2	4.4 ± 0.2		<0.001	<0.001	
Plasma progesterone (ng/ml)	6.1 ± 1	4.9 ± 0.7		10.4 ± 1	4.9 ± 0.5*		13.9 ± 1	6.9 ± 0.8*		<0.001	<0.001	
Fetal measurements												
Fetal weight (g)	10	9		12	10		12	10		–	–	
Fetal weight (g)	19 ± 0.4	20 ± 0.4		663 ± 23	732 ± 19*		4465 ± 120	3593 ± 192*		<0.001	<0.001	
Brain weight (g)	0.89 ± 0.04	0.89 ± 0.04		13.7 ± 0.3	14.7 ± 0.3*		42.5 ± 0.6	40.2 ± 0.8		0.308	<0.001	
Peri-renal fat weight (g)	–	–		2.2 ± 0.2	2.4 ± 0.2		20 ± 2	18 ± 1		0.505	<0.001	
Liver weight (g)	1.5 ± 0.1	1.7 ± 0.1		44 ± 2	46 ± 2		141 ± 9	117 ± 8		0.131	<0.001	
Pancreas weight (g)	–	–		0.6 ± 0.1	0.8 ± 0.1		3.6 ± 0.2	3.2 ± 0.3		0.396	<0.001	
Brain:fetal weight (g:kg)	47 ± 1	45 ± 2		21 ± 1	20 ± 1		9.6 ± 0.2	11.4 ± 0.5*		0.715	<0.001	
Amniotic glucose (μmol/ml)	1.54 ± 0.15	2.02 ± 0.11*		0.91 ± 0.05 ^a	1.17 ± 0.05*		0.16 ± 0.03	0.08 ± 0.02*		<0.001	<0.001	
Plasma glucose (μmol/ml)	–	–		0.33 ± 0.06	0.45 ± 0.06*		0.40 ± 0.06	0.24 ± 0.04*		0.726	<0.001	
Plasma insulin (IU/ml)	–	–		10.3 ± 0.5	10.0 ± 0.5		12.2 ± 1.4 ^b	10.2 ± 1.7 ^a		0.292	0.168	
Plasma IGF1 (pmol/ml)	–	–		23.7 ± 1.0	24.4 ± 1.4		33.0 ± 3.2	31.0 ± 3.5		0.787	<0.004	

Mean comparisons for diet (C versus H) within day presented for significant interactions ($P \leq 0.05$) only. Values are means \pm S.E.M. *Differs from control (C) within day, $P < 0.05$. ^aN = 1; ^bN = 2.

concentrations. Fetal plasma insulin and IGF1 concentrations at both day 90 and 130 were highly variable and were unaffected by maternal diet.

Placental growth and vascular development

Changes in placental growth and vascular development resulting from maternal dietary treatment and stage of gestation are presented in Table 2. Gravid uterine weight and combined amniotic and allantoic fluid volume remained similar between dietary groups up to day 90 of gestation, but declined in the H versus the C group by day 130, resulting in a significant interaction between stage of gestation and maternal nutrition. Total and mean placentome weights in the H group were significantly reduced compared with the C group, but predominantly during late gestation, also resulting in a significant stage \times nutrition interaction. At day 130 of gestation, this represented a reduction in total and mean placentome weight of 46 and 41% respectively, in the H relative to the C group ($P < 0.01$). Placentome number was not influenced by maternal diet or stage of gestation. The fetal:placentome weight ratio increased as gestation advanced in both treatment groups and was greater in H compared with C beginning on day 90. Overall, cellular proliferation of the fetal cotyledon tended ($P = 0.072$) to be less in H versus C (Table 2) and decreased across gestation.

Over all stages of gestation, cotyledonary capillary surface density was greater in H compared with C and increased in both groups across gestation (Table 2). At day 50 of gestation, area per capillary and capillary area density was reduced in the fetal cotyledon of H compared with C fetuses (Table 2). These early reductions in cotyledonary capillary area density and area per capillary are readily observed (see micrographs, Fig. 2) and led to significant interactions between maternal dietary intake and stage of gestation (Table 2). As gestation advanced to day 90, cotyledonary capillary number density in the H group (Table 2) increased compared with controls. As gestation advanced to day 130, cotyledonary capillary surface, area, and number densities increased irrespective of diet, whereas area per capillary in the fetal cotyledon declined.

Proliferative growth in the maternal caruncle was not significantly altered by diet, but this parameter did remain numerically lower at all stages of gestation in H versus C (Table 2). As gestation advanced, maternal caruncular cellular proliferation declined. None of the vascularity parameters in the maternal caruncle were altered by diet. However, similar to the fetal cotyledon, capillary surface density, capillary area density and capillary number density increased as gestation advanced, whereas area per capillary declined.

Table 2 Placental growth and vascular development in control (C) and high (H) intake pregnancies at necropsy on day 50, 90 and 130 of gestation.

	Day 50 of gestation		Day 90 of gestation		Day 130 of gestation		P value	Stage	Interaction
	C	H	C	H	C	H			
N	10	10	12	10	12	10			
Gravid uterus weight (kg)	0.61 ± 0.02	0.62 ± 0.02	2.73 ± 0.15	2.71 ± 0.09	7.84 ± 0.32	6.45 ± 0.31*	<0.001	<0.001	<0.002
Fetal fluid volume (ml)	150 ± 15	155 ± 16	928 ± 81	964 ± 52	1514 ± 158	1256 ± 94	0.351	<0.001	0.226
Total placentome weight (g)	144 ± 22	124 ± 8	607 ± 50	520 ± 46	548 ± 39	293 ± 24*	<0.001	<0.001	<0.009
Mean placentome weight (g)	1.4 ± 0.1	1.3 ± 0.1	5.7 ± 0.4	4.8 ± 0.3	5.4 ± 0.4	3.2 ± 0.5*	<0.001	<0.001	<0.002
Placentome number	100 ± 6	101 ± 4	107 ± 3	107 ± 4	101 ± 3	99 ± 8	0.917	0.326	0.971
Fetal:placentome ratio (g/g)	0.15 ± 0.01	0.16 ± 0.01	1.16 ± 0.09	1.51 ± 0.13*	8.41 ± 0.37	12.46 ± 0.39*	<0.001	<0.001	<0.001
Cotyledon N	10	10	12	10	12	10			
Cellular proliferation (%)	15.7 ± 1.3	13.5 ± 0.7	6.8 ± 0.2	6.2 ± 0.5	5.8 ± 0.5	5.5 ± 0.4 ^a	<0.072	<0.001	0.347
Capillary surface density (µm/µm ²)	0.016 ± 0.003	0.018 ± 0.004	0.010 ± 0.001	0.019 ± 0.004	0.065 ± 0.003	0.074 ± 0.003	<0.001	<0.001	0.537
Area per capillary (µm ²)	554 ± 95	273 ± 31*	128 ± 17	100 ± 21	135 ± 11	166 ± 20	<0.001	<0.001	<0.001
Capillary area density (%)	6.9 ± 1.0	3.3 ± 0.5*	3.3 ± 0.5	4.5 ± 0.6	15.5 ± 1.0	17.4 ± 0.9	0.960	<0.001	<0.005
Capillary no. density (× 10 ⁶)	192 ± 40	161 ± 24	287 ± 28	588 ± 79*	1230 ± 42	1157 ± 75	0.118	<0.001	<0.001
Caruncle N	9	10	11	9	12	10			
Cellular proliferation (%)	7.3 ± 0.8	6.7 ± 0.9	1.5 ± 0.2	1.0 ± 0.1	1.1 ± 0.2	0.9 ± 0.2 ^a	0.302	<0.001	0.893
Capillary surface density (µm/µm ²)	0.038 ± 0.004	0.030 ± 0.004	0.057 ± 0.010	0.071 ± 0.009	0.114 ± 0.005	0.117 ± 0.004	0.581	<0.001	0.274
Area per capillary (µm ²)	442 ± 48	424 ± 49	240 ± 25	214 ± 21	258 ± 20	343 ± 28	0.609	<0.001	0.169
Capillary area density (%)	18.1 ± 2.1	13.9 ± 2.2	18.4 ± 3.2	21.8 ± 2.7	40.7 ± 2.2	47.4 ± 1.9	0.330	<0.001	0.086
Capillary no. density (× 10 ⁶)	473 ± 72	376 ± 45	912 ± 177	1295 ± 208	1795 ± 82	1591 ± 107	0.795	<0.001	0.057

Mean comparisons for diet (C versus H) within day presented for significant interactions ($P \leq 0.05$) only. Values are means \pm s.e.m. *Differs from control (C) within day, $P < 0.05$. N, number of ewes. ^aN = 2.

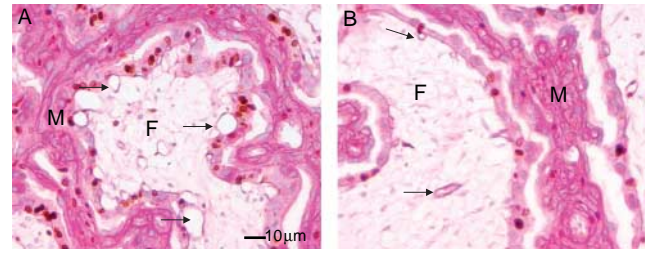


Figure 2 Representative micrographs of vascular perfused cotyledonary tissue at day 50 of gestation in control (A) and high (B) dietary-intake groups. The pinkish staining (periodic acid-Schiff's reagent) represents primarily basement membranes, including those of the microvessels (capillaries, arterioles, and venules; Borowicz *et al.* 2007), the brown nuclei indicate the presence of BrdU and therefore marks DNA synthesis and thus cell proliferation (Johnson *et al.* 1997a, 1997b, Reynolds *et al.* 1998), and the bluish nuclei represent non-proliferating nuclei counterstained stained with hematoxylin. Cotyledonary capillary area density (capillary area per unit of cotyledonary tissue) and number density (number of capillaries per unit of cotyledonary tissue) were reduced by 46 and 51% respectively in the high (B) versus the control (A) dietary groups at day 50 of gestation (Table 2). Arrows show examples of cotyledonary capillaries. Note the difference in both capillary size and number between panels A and B (C versus H respectively). M = maternal caruncular (dark pinkish), and F = fetal cotyledonary (light pinkish) tissue areas. Bar (A) represents both panels = 10 µm.

Discussion

The design of the present study enabled cross-sectional assessment of fetoplacental growth at three key stages of gestation in a large cohort of adolescent animals where embryo donor genetics was controlled for at conception. This approach was critical to determine the basis of the previously observed changes in placental development and function in late gestation. The study demonstrated that placental mass *per se* is not perturbed by maternal overfeeding until sometime during the final third of pregnancy and as such confirms previous studies in which the pregnancies were terminated at single points coincident with either mid (Wallace *et al.* 2004b, Redmer *et al.* 2005) or late (Wallace *et al.* 2000, 2002a, 2002b, 2007) gestation. Moreover, the data revealed for the first time that the fetuses of over-nourished dams may be initially on a higher growth trajectory than control fetuses between day 50 and 90 of gestation, with fetal weight being 11% greater by day 90 in the H group. Indeed, the greater fetal amniotic glucose at day 50 and the greater amniotic and fetal plasma glucose at day 90 are commensurate with the ready availability of nutrients in the maternal circulation of the overnourished dams.

Under normal conditions (i.e., normal placental growth and development), transport of glucose to the fetus is dependent on the maternal:fetal transplacental glucose concentration gradient (Bell *et al.* 1999), which is the physiological driving force that determines placental glucose uptake and transfer to the fetus

(Simmons *et al.* 1979). In the present study, maternal glucose concentrations were elevated at all necropsy stages in the overnourished dams, and it was only during the last third of gestation, when the mass of the placenta was subsequently impaired, that the absolute nutrient transfer capacity of the placenta became limiting for further fetal growth (Wallace *et al.* 2002b). Consequently, by day 130 of gestation, fetal growth had slowed and fetal body weight was reduced by 20% in fetuses from overnourished dams compared with those from controls, coincident with a 40% reduction in placental mass. This switching between prenatal growth trajectories in response to a putative relative excess and then a gradual deficit of glucose supply (the main fetal fuel), may underlie previous observations that reported increased fetal weight-specific perirenal and carcass fat contents at late gestation in growth-restricted fetuses from overnourished dams (Matsuzaki *et al.* 2006).

The design of the present study also allowed us for the first time to assess early placental growth and vascular development in relation to maternal nutritional status. Although cellular proliferation within the fetal cotyledon was only marginally influenced by maternal diet at day 50 of gestation, we did observe a significant reduction in the vascular development within the fetal cotyledon at this early stage. The reductions in capillary area density and area per capillary (i.e., vessel size) in the overnourished dams may explain the previously reported reductions in uterine blood flow in mid-gestation (Wallace *et al.* 2008a) and in uteroplacental blood flows and nutrient uptakes in late gestation (Wallace *et al.* 2002a, 2002b, 2003a) in this experimental paradigm. The mechanism underlying this nutritionally-mediated alteration in vascular development is unknown. One possibility is that nutritionally induced suppression of the major sex steroids in the overnourished dams may be influencing both placental angiogenesis and vascular development. In this and earlier studies, we have shown that maternal progesterone concentrations are attenuated throughout gestation in overnourished dams (Wallace *et al.* 1997b, 2003a). We had insufficient plasma to evaluate maternal estrogen in the present study due to the requirement to extract the steroid from a large volume of plasma. However, we have recently shown that circulating oestradiol-17 β concentrations in identically nutritionally manipulated overnourished dams fail to increase in parallel with those of control dams between day 50 and 75 of gestation (Wallace *et al.* 2008b). The reproductive steroids are strongly implicated in the control of uterine growth (Johnson *et al.* 1997a, 1997b, Reynolds *et al.* 1998), vascular development and function, and blood flow (Cullinan-Bove & Koos 1993, Reynolds & Redmer 2001, Reynolds *et al.* 2006). In addition, physiological doses of oestradiol upregulate expression of angiogenic factors in the uterine endometrium (Cullinan-Bove & Koos 1993, Reynolds *et al.* 1998, Johnson *et al.* 2006),

which provides a plausible route whereby nutrition may affect steroid levels and ultimately vascular architecture and function in the placenta.

Alternatively, nutritionally-mediated alterations in placental angiogenesis and vascular development may reflect differences in circulating somatotrophic hormones. Insulin and, to a lesser extent, IGF1 concentrations are elevated from early in gestation in overnourished dams and provide a sustained anabolic stimulus to maternal tissue deposition (Wallace *et al.* 1997a, present study). The resulting progressive increase in adiposity may compromise blood flow to the gravid uterus and hence limit placental vascular development. Maternal cardiac output during pregnancy normally increases by ~70% in the sheep, which results in a redistribution of the percentage of cardiac output going to the various organs, and particularly the gravid uterus (Rosenfeld 1977). Clearly, both cardiac output and the partitioning of blood to the maternal versus gravid uterine tissues may be influenced by the increasing adiposity of the dams, but this aspect has not yet been directly measured in the overnourished paradigm. Irrespective of the putative mechanism, the early perturbation in fetal rather than maternal placental vascular development in overnourished pregnancies is in line with previous observations of a relative delay in ovine placental lactogen secretion in this model (indicative of inadequate binucleate cell migration from the fetal trophoctoderm; Lea *et al.* 2007) and with reduced proliferative activity within the fetal trophoctoderm at the apex of placental growth (Lea *et al.* 2005).

However, the early reduction in fetal cotyledonary vascular development was not evident at mid-gestation (day 90). Indeed, at this stage, a twofold increase in capillary area density was observed in the fetal cotyledon of overnourished dams, whereas all other placental vascular parameters were independent of maternal nutrition. Although this contrasts with the observations at day 50, it may reflect a transient compensatory vascular response and an acceleration (or mismatch) of the normal pattern of placental vascular development at this stage, before the mass of the placenta *per se* is perturbed. However, notwithstanding these observations, serial assessments of uterine arterial blood flow *in vivo* reveal a 40% reduction at day 88 of gestation in overnourished compared with control dams (Wallace *et al.* 2008a). By late gestation, total placentome mass was reduced by 46% yet placental vascular morphology assessed by the techniques described herein was similar in both groups. This suggests that the major reductions in uterine and umbilical blood flows previously measured in late gestation (Wallace *et al.* 2002a, 2002b) may be more reflective of a total reduction in total placental vascular volume due to reduced placental size *per se* rather than reduced vascular volume per unit of placental tissue (as measured here). Indeed, when late gestation uterine

blood flow is expressed on a placental weight-specific basis, it is equivalent in overnourished and control pregnancies (Wallace *et al.* 2002a, 2002b).

Although it can be argued that the morphometric methodologies used herein are limited by the 2-D nature of the image analyses, the changes in the patterns of vascular development throughout gestation are strikingly similar to those reported previously during normal pregnancy in adult ewes using identical procedures (Borowicz *et al.* 2007). Furthermore, both sets of data are similar to the observations of Stegeman (1974) both in pattern and magnitude of the increase in placental vascularization that she determined using a microscopic point counting method. Moreover, the increases in placental capillary area density and in cotyledonary capillary number and surface density from early to late gestation are in broad agreement with previously documented cross-sectional measurements of changes in uterine (maternal) and umbilical (fetal) blood flows *in vivo*, measured at mid and late gestation (Rosenfeld *et al.* 1974, Molina *et al.* 1990).

In this study, we evaluated fetoplacental growth and vascular development at several critical stages of gestation using histology combined with sophisticated image analysis techniques. We determined that high maternal intake designed to promote rapid maternal growth influenced capillary development in the fetal portion of the placenta during early to mid-pregnancy, which may underlie the subsequent reduction in placental mass and hence fetal nutrient supply observed during the final third of gestation. These data will guide future studies designed to determine the mechanisms responsible for compromised placental development and function in overfed adolescents.

Materials and Methods

Animals and experimental design

All procedures were licensed under the UK Animals (Scientific Procedures) Act of 1986 and approved by the Rowett Institute's Ethical Review Committee.

Embryos from superovulated adult ewes (Border Leicester × Scottish Blackface), inseminated by a single sire, were recovered on day 4 after estrus and transferred synchronously in singleton into the uterus of recipient ewe lambs (Dorset Horn × Greyface), exactly as described previously (Wallace *et al.* 1997a). This protocol ensured that placental and fetal growth was not influenced by varying fetal number or partial embryo loss. In addition, the use of a single sire and a limited number of embryo donors maximized the homogeneity of the resulting fetuses. Donor ewes were multiparous, between 3 and 4 years of age, had a body weight of 72.9 ± 2.32 kg and had a body condition score of 2.4 ± 0.03 units (evaluated on a scale of 0 to 5, where 0 = extremely emaciated and 5 = extremely fat, according to the criteria of Russel *et al.* (1969)) at the time of embryo recovery. Embryo transfers were carried out in two batches of three and four consecutive days

separated by a two-week period during the mid-breeding season of the same year. As far as possible, pregnancies resulting from each embryo transfer day were equally represented at each of the three gestational stages studied. All animals were housed in individual pens under natural lighting conditions.

At the time of embryo transfer, recipient ewe lambs were peripubertal (~7.5 months of age) and had a mean body weight of 43.7 ± 0.39 kg, body condition score of 2.3 ± 0.01 units, and ovulation rate of 2.1 ± 0.15 . Based on these parameters, recipient ewe lambs were evenly allocated to receive a control (C) or high (H) quantity of the same complete diet. Care was also taken to randomize for the maternity of the embryo so that embryo donor genetics were equally represented in both groups at the outset of the study. The dietary level in the C group was calculated to maintain normal maternal adiposity throughout gestation (i.e., no change from initial starting adiposity) and hence meet the estimated metabolizable energy requirements for optimum conceptus growth and pregnancy outcome in this genotype (AFRC 1993). To achieve this objective, the moderate intake, C group, was fed to promote a modest maternal weight gain (~50 g/day) during the first two thirds of gestation, followed by step-wise increases in maternal intake during the final third of gestation that were calculated to meet the increasing demands of the developing fetus. By contrast, the H or *ad libitum* intake group consumed equivalent to approximately twice the estimated metabolizable energy requirements, and this level of intake was calculated to promote rapid maternal growth. The complete diet supplied 12 MJ of metabolizable energy and 140 g of crude protein per kg and was offered in two equal feedings at 0800 and 1600 h daily (see Wallace *et al.* 2006b for full details of the diet composition, analyses, and typical intakes for this nutritional paradigm).

Maternal body condition score was evaluated every two weeks by the same experienced assessor. Pregnancy status was determined by transabdominal ultrasonography at approximately day 45 of gestation (gestation length ~145 days). Pregnancy was established and maintained in 34 and 30 ewes in the C and H groups respectively. Pregnancies were terminated at day 50, 90 or 130 of gestation ($n=10$ to 12 pregnancies per treatment group at each day of gestation). At necropsy on day 50, one animal from the H group was diagnosed as having severe ruminitis, and maternal and fetal necropsy data collected from this ewe were excluded from further analysis.

Blood sample collection and necropsy procedures

Before necropsy on day 50, 90 or 130 of gestation, a catheter was inserted into the maternal jugular vein and a blood sample was collected. Exactly 1 h before euthanasia, to determine the relative rate of placental cell proliferation (the labeling index), the dams were infused with 5-bromo-2'-deoxyuridine (BrdU, 5 mg/kg of body weight, dissolved in phosphate buffered saline (PBS) to form a saturated solution of 16.7 mg of BrdU/ml, pH 7.0), as we have previously described (Jablonka-Shariff *et al.* 1993). Euthanasia was achieved by i.v. administration of an overdose of sodium pentobarbitone (20 ml Euthesate; 200 mg

pentobarbitone/ml; Willows Francis Veterinary, Crawley, UK) and exsanguination (by severing the main vessels of the neck). The gravid uterus was removed, weighed, and opened, and a sample of amniotic fluid was rapidly obtained for subsequent glucose analyses. At day 90 or 130 of gestation all fetal hearts were clearly and strongly beating when the uterus was opened and a blood sample was collected by cardiac puncture immediately before intracardiac administration of sodium pentobarbitone (3 ml Euthesate) to kill the fetus. After clamping the umbilical cord, the fetus was removed, dried, weighed, and its component organs were dissected. The maternal liver, pancreas, and perirenal fat were also dissected and weighed.

Maternal and fetal plasma and amniotic glucose concentrations at necropsy were determined in duplicate using a Yellow Springs Instruments (YSI, Yellow Springs, OH, USA) dual biochemistry analyzer (model 2700), as previously described (Wallace *et al.* 2002a). Variation between duplicates was <5% in all cases. Maternal progesterone, and maternal and fetal insulin and IGF1 concentrations were measured in duplicate by RIA, as described previously (Ronayne & Hynes 1990, Bruce *et al.* 1991, MacRae *et al.* 1991). The sensitivities of the assays were 0.2 ng progesterone/ml, 4 μ U insulin/ml, and 6 pmol IGF1/ml. The concentration of each hormone was measured within a single assay and the intraassay coefficients of variations were <10%.

Quantification of placental cellular proliferation and vascularity

Two representative placentomes from the gravid uterine horn were sliced into 7 mm cross sections and were immersion fixed in Carnoy's fixative for 6 h, followed by 70% ethanol, which changed once after 24 h (for BrDU immunohistochemistry).

Maternal caruncular and fetal cotyledonary tissues of the placenta were perfused after catheterizing branches of the uterine and umbilical arteries respectively, as previously described (Borowicz *et al.* 2007). Briefly, a catheter was inserted into the artery, secured with suture and perfused in the following sequence: 1) 10 ml PBS; 2) 5 ml or less of Evans Blue dye to check perfusion; 3) 10 ml PBS; 4) 10 ml Carnoy's solution; 5) 10 ml PBS and 6) 10.8 ml of a Mercox resin vascular casting mixture (Ladd Research Industries, Inc., Burlington, Vermont, USA). Perfused placentomes were then removed from the uterus, weighed, sliced into 7 mm cross sections and immersed in Carnoy's fixative. All remaining placentomes were dissected and weighed.

To evaluate cellular proliferation and vascularity, Mercox-perfused placentome tissues (one sample each from maternal caruncular and fetal cotyledon perfusions) were embedded in paraffin, sectioned (5 μ m) and then were incubated with a monoclonal antibody to BrDU (a thymidine analog; 100 μ g/ml; Roche Diagnostics Ltd) or normal mouse IgG (2 μ g/ml, negative control), followed by the previously described staining method for determination of vascularity (Reynolds *et al.* 1992). Thereafter, placentome vascularity and cellular proliferation were quantified according to the methods of Borowicz *et al.* (2007). Briefly, the slides were visualized with a Leica DMR microscope (Leica

Microsystems, Welzlar, Germany) at 20 \times magnification and 10 digital images of well-perfused regions were captured with a Hamamatsu camera (C5810, Leica Microsystems) and analyzed using Image-Pro Plus, version 4.5.1 software (Media Cybernetics, Inc., Silver Spring, MD, USA). The number of BrDU-labeled nuclei was counted and divided by the total number of nuclei in the maternal caruncular or fetal cotyledonary tissue. Figure 2 shows placentome vascularity and BrDU staining. Total caruncular area (μm^2) and shrinkage area (μm^2) were determined, and each caruncular or cotyledonary capillary was individually traced to quantify the total vessel area (μm^2), number, and perimeter (μm) per each tissue area. Shrinkage was determined by tracing all areas of shrink space (visual artifactual separation of tissue areas seen in most sections) within an image and subtracting from the total tissue areas as shown below. Based on these measurements, total cotyledonary area (μm^2 ; total tissue area – (caruncular area + shrinkage area)), capillary area density (%; capillary area/tissue area), capillary number density (number of capillaries per mm^2 ; capillary number/tissue area), capillary surface density (capillary perimeter/tissue area), area per capillary (μm^2 ; capillary area/capillary number), and perimeter per capillary (μm ; capillary perimeter/capillary number) were calculated for each perfused cotyledon and caruncle image.

Statistical analysis

Data are presented as means \pm S.E.M. Differences between nutritional treatments for maternal and fetal blood parameters and organ weights from each of days 50, 90 or 130 of gestation were analyzed by one-way ANOVA (General Linear Models, Minitab, State College, PA, USA). A similar approach was used to analyze body weight and body condition score collected throughout gestation after calculating a mean individual value spanning the first (from day 4 to day 49), second (from day 50 to day 90), and final third of gestation (day 91 to day 130). Uteroplacental mass and placental vascularity data from days 50, 90, and 130 of gestation were analyzed by two-way ANOVA (PROC GLM procedure of SAS; SAS Inst. Inc., Cary, NC, USA) to determine the main effects of nutrition and day of gestation and the interaction between nutrition and day of gestation. In specified instances, these latter parameters were also analyzed by one-way ANOVA within a day of gestation. When the nutrition \times day of gestation interaction was significant ($P < 0.05$), means were separated by orthogonal contrasts of the least squares means. Main effects were considered significant when $P < 0.05$.

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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