Endocr (2008) 33:53–61
DOI 10.1007/s12020-008-9052-3

ORIGINAL PAPER

Placental vascularity and growth factor expression in singleton, twin, and triplet pregnancies in the sheep

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Received: 17 December 2007 / Accepted: 4 March 2008 / Published online: 8 April 2008

Abstract For singleton, twin, and triplet pregnancies, uteri were collected on day 140 of pregnancy. For each ewe ($n = 18$), placentomes were fixed by arterial perfusion supplying the fetal (cotyledon) and maternal placenta (caruncle). Tissue sections were stained for determination of vascularity by image analysis. Further, protein expression for factor VIII, vascular endothelial growth factor (VEGF) and its receptor, VEGFR1, as well as basic fibroblast growth factor (FGF2) and its receptor, FGFR, in tissue sections was determined by immunohistochemistry and image analyses. Cotyledonary and caruncular samples were analyzed for expression of mRNA for *Vegf* and its two receptors, *Vegfr1* and *Vegfr2*, as well as *Fgf2* and *Fgfr*. Fetal number did not affect placental capillary density or factor VIII expression, whereas increased fetal number reduced total cotyledon and caruncle capillary volume. While expression of *Vegf*, *Vegfr1*, *Vegfr2*, and *Fgfr* mRNA in cotyledonary but not caruncular tissue was greater in twin pregnancies compared to singleton and triplet pregnancies, protein expression of VEGF in the placentome decreased with increasing numbers of fetuses, VEGFR1 did not change, and FGFR was greater in twin versus singleton and triplet pregnancies. Fetal number did not affect the expression of *Fgf2* mRNA in placental tissues, whereas FGF2 protein expression was less in triplet compared to singleton and twin pregnancies. Reduced fetal and placental weights in twins and/or triplet pregnancies are associated with an overall decrease in total placental vascularity, VEGF and FGF2 and/or FGFR protein expression, but not in angiogenic factor mRNA expression or VEGFR1 protein expression in sheep.

Keywords Placental vascularity · Angiogenic factors · Multiple pregnancies · Sheep

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Introduction

The incidence of multiple pregnancies continues to increase with the increased participation in artificial reproductive technologies in humans [1]. Compared to singleton

pregnancies, multiple pregnancies result in an alteration in both maternal and fetal physiology. In sheep, increased numbers of fetuses was associated with reduced uterine blood flow per fetus [2, 3], decreased placentome numbers and total placentome weight per fetus [4–6]; decreased fetal weight [6–11], and increased neonatal mortality [12]. If lambs from multiple pregnancies survive the neonatal period, there is a decrease in thermoregulation [13–15], potential alterations in glucose metabolism, increased chances for obesity and hyperlipidemia as well as other endocrine and structural abnormalities [8, 11, 12, 16, 17].

Uterine capacity, defined as the ability to successfully carry a fetus to term, is determined by both maternal (i.e., uterine size) and conceptus components. For the conceptus, the size and nutrient transfer capacity of the placenta plays a key role in determining the prenatal growth trajectory of the fetus and hence, directly affects birth weight [18–26]. Placental growth is regulated by numerous factors such as hormones and growth factors, including such key angiogenic factors as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF2). Expression of VEGF and FGF2 has been shown to increase during vascular development in the sheep placenta [27–30]. Vascular endothelial growth factor promotes cell proliferation, differentiation, permeability, vascular tone, and stimulates production of other vasoactive molecules such as nitric oxide [31–34]. Vascular endothelial growth factor binds to one of two receptors: VEGFR1, which may maintain vascular integrity; and VEGFR2, which is considered to be the major stimulator of angiogenesis [35, 36]. Basic fibroblast growth factor promotes cell proliferation, differentiation and migration, and tissue remodeling when it binds to one of its high affinity receptors, specifically, FGFR3IIIc (FGFR; [27, 37, 38]).

While blood flow has been demonstrated to be reduced to each fetus in ewes carrying multiple fetuses [2], data on placental vascularity or expression of angiogenic factors are very limited. We hypothesized that with an increase of fetal number within the uterus placental capillary vascularity will be enhanced to compensate for reduced placental weight per fetus, and the expression of angiogenic factors in the placenta will depend on the number of fetuses. Therefore, our objective was to determine vascularity as well as mRNA expression for *Vegf*, *Vegfr1*, *Vegfr2*, *Fgf2*, and *Fgfr* and protein expression for factor VIII, VEGF, VEGFR1, FGF2, and FGFR in the placentomes on day 140 of gestation in ewes carrying singletons, twins or triplets.

Results

Fetal and placental weights decreased ($P < 0.05$) in twin and triplet pregnancies compared to singleton pregnancies

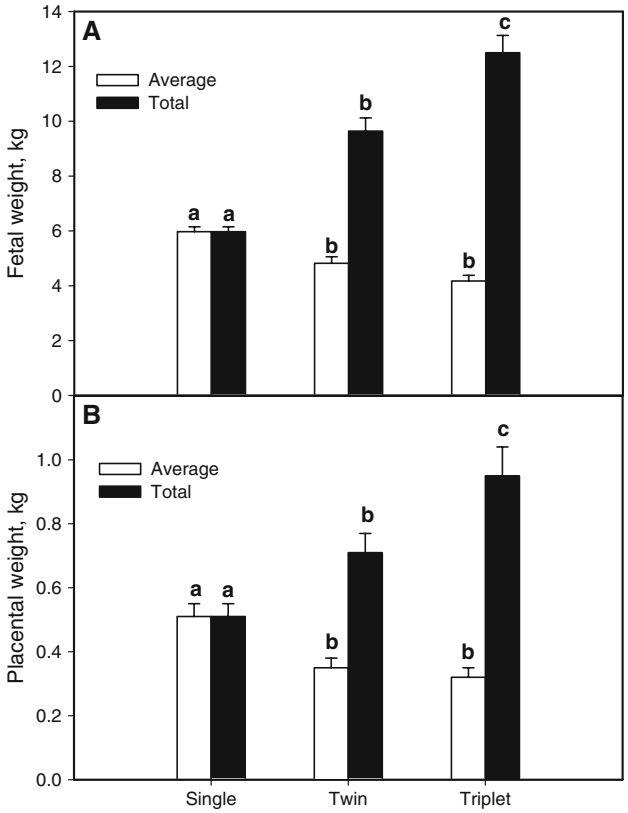

Fig. 1 Average and total fetal (**a**) and placental (**b**) weights from singleton, twin and triplet pregnancies on day 140 of gestation. [a,b]Means ± SEM within a measure differ, $P < 0.05$. (Adapted from Grazul-Bilska et al. [6])

(Fig. 1); however, there was no difference in average fetal or placental weights between twin and triplet pregnancies. Total fetal and placental weight increased ($P < 0.05$) as numbers of fetuses increased (Fig. 1). However, placental efficiency, expressed as the ratio of fetal weight to placental weight, was similar for singleton, twin, and triplet pregnancies (12.2 ± 0.9, 14.0 ± 1.0, 13.4 ± 1.6, respectively).

Number of fetuses in the uterus did not affect capillary area density, capillary number density, capillary surface density or mean capillary area in either caruncular or cotyledonary tissues (Table 1). Further, there was no difference in expression of factor VIII, an endothelial cell marker, within a tissue for singleton, twin or triplet pregnancies (percentage area stained was 2.1 ± 0.1, 1.9 ± 0.1 and 2.1 ± 0.2, respectively). When adjusted for total placental volume, total capillary volume per fetus in twin and triplet pregnancies tended ($P = 0.09$) to be less in the cotyledon, and was less ($P = 0.01$) in the caruncle, compared to singletons pregnancies (Table 1). Cotyledonary and caruncular weight per fetus was less ($P < 0.03$) in multiple compared to singleton pregnancies (Table 1).

Table 1 Caruncular and cotyledonary weights and capillary measurements from singleton, twin and triplet pregnancies

	Singleton	Twin	Triplet	*P* value
Number of ewes	8	7	3	
Cotyledon				
Capillary area density	31.4 ± 1.4	32.1 ± 1.5	30.8 ± 2.3	0.88
Capillary number density (#/μm^2)	3532 ± 330	3472 ± 352	2300 ± 539	0.16
Capillary surface density (μm/μm^2)	8868 ± 245	9047 ± 261	8810 ± 399	0.84
Mean area per capillary (μm^2)	100 ± 16	103 ± 17	145 ± 26	0.34
Total capillary volume per fetus (ml)	55.8 ± 0.6^a	35.5 ± 0.7^b	25.2 ± 1.0^b	**0.09**
Cotyledonary weight per fetus (g)	182.3 ± 21.2^a	109.1 ± 22.7^b	81.4 ± 34.7^b	**0.03**
Caruncle				
Capillary area density	48.1 ± 3.1	48.1 ± 2.3	50.2 ± 2.5	0.89
Capillary number density (#/μm^2)	1207 ± 102	1421 ± 174	952 ± 127	0.17
Capillary surface density (μm/μm^2)	2284 ± 78	2335 ± 167	2081 ± 109	0.49
Mean area per capillary (μm^2)	470 ± 60	420 ± 51	574 ± 91	0.37
Total capillary volume per fetus (ml)	67.8 ± 5.1^a	45.1 ± 5.5^b	41.3 ± 7.7^b	**0.01**
Caruncular weight per fetus (g)	140.7 ± 9.8^a	95.1 ± 10.6^b	82.2 ± 15.0^b	**0.01**

^{a,b} Means ± SEM within a row differ

Bold values are statistically significant *P* values for specific measurements

While caruncular *Vegf*, *Vegfr1*, and *Vegfr2* mRNA expression were similar in singletons, twin or triplet pregnancies, cotyledonary *Vegf*, *Vegr1*, and *Vegfr2* mRNA expression was enhanced ($P < 0.05$) in twin pregnancies compared to singleton and triplet pregnancies (Fig. 2). While there was no difference of *Fgf2* mRNA expression in either caruncular or cotyledonary tissue across pregnancy types, expression of *Fgfr* mRNA was greater ($P < 0.05$) in cotyledonary tissue from twin pregnancies than in cotyledonary tissue from singleton and triplet pregnancies (Fig. 3). Expression of *Fgfr* mRNA in caruncular tissue was similar for singleton, twin and triplet pregnancies (Fig. 3).

Expression of VEGF, VEGFR2, FGF2, and FGFR protein was detected in caruncular and cotyledonary tissues. However, the localization of VEGF, VEGFR2, as well as FGF2 and its FGFR protein in caruncular tissues differed from cotyledonary tissues. In the cotyledon, VEGF was localized in the walls of the major fetal arterioles and capillaries that were greater than 15 μm in diameter and was not present in capillaries less than 10 μm (Fig. 4a). In the caruncle, VEGF staining was present in the walls of most of the blood vessels (Fig. 4b). Positive staining for VEGFR2 was present in the walls of the caruncular blood vessels of all sizes, and in the cotyledon, was present only in binucleate cells (Fig. 4b). Positive staining for FGF2 was localized exclusively in the walls of caruncular blood vessels smaller than 15 μm in diameter (Fig. 4c and e), whereas major caruncular blood vessels remained unstained, and no FGF2 staining was present in cotyledon. Positive, punctuate, scattered FGFR staining was detected in both caruncular and cotyledonary tissues (Fig. 4d).

Protein expression of VEGF was greatest ($P < 0.001$) in placentomes from singleton, less from twin, and least from triplet pregnancies (Fig. 5). The protein expression of VEGFR2 was similar ($P = 0.94$) among placentomes of singletons, twins and triplets (Fig. 5).

While FGF2 protein expression tended to decrease ($P = 0.10$) in placentomes from triplet pregnancies compared to placentomes from singleton and twin pregnancies, FGFR protein expression tended to be greater ($P = 0.07$) in placentomes from twin pregnancies compared to singleton and triplet pregnancies (Fig. 6).

Discussion

In the present study, total caruncular and cotyledonary vascular volume was reduced with increasing numbers of fetuses. However, there were no differences in any of the placental capillary vascularity measurements in singleton to compare with multiple pregnancies. Therefore, we were unable to prove our hypothesis that placental capillary vascularity increases with increasing numbers of fetuses. This indicates that by the end of gestation a synchronous reduction of both the fetus and the placenta has occurred as number of fetuses increased. While it is unknown in the current study when this reduction in both fetal and placental sizes may have occurred, it has been reported that individual fetal weight gain begins to decrease with

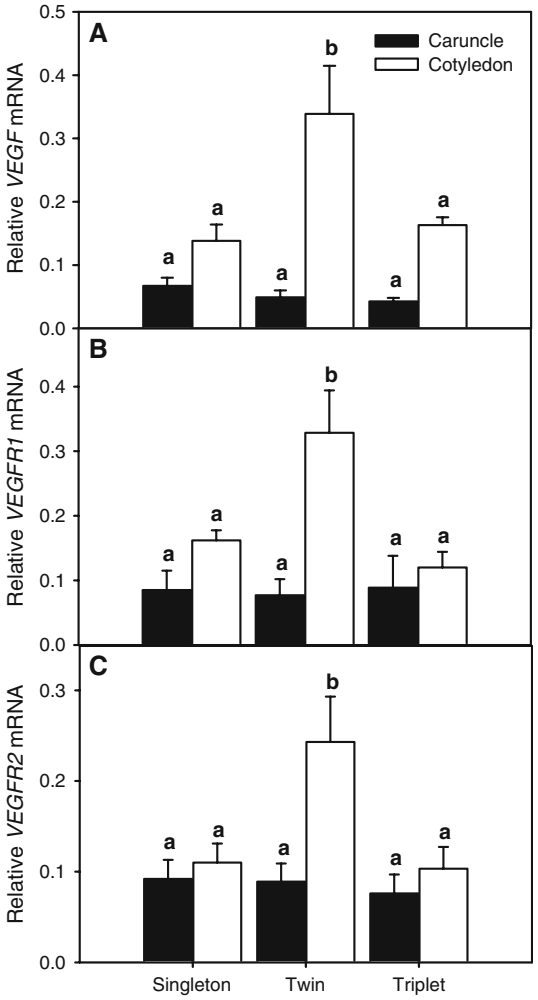

Fig. 2 Caruncular and cotyledonary mRNA expression of *VEGF* (**a**), *VEGFR1* (**b**), and *VEGFR2* (**c**) from singleton, twin and triplet pregnancies on day 140 of gestation. [a,b]Means ± SEM within a tissue differ, $P < 0.05$

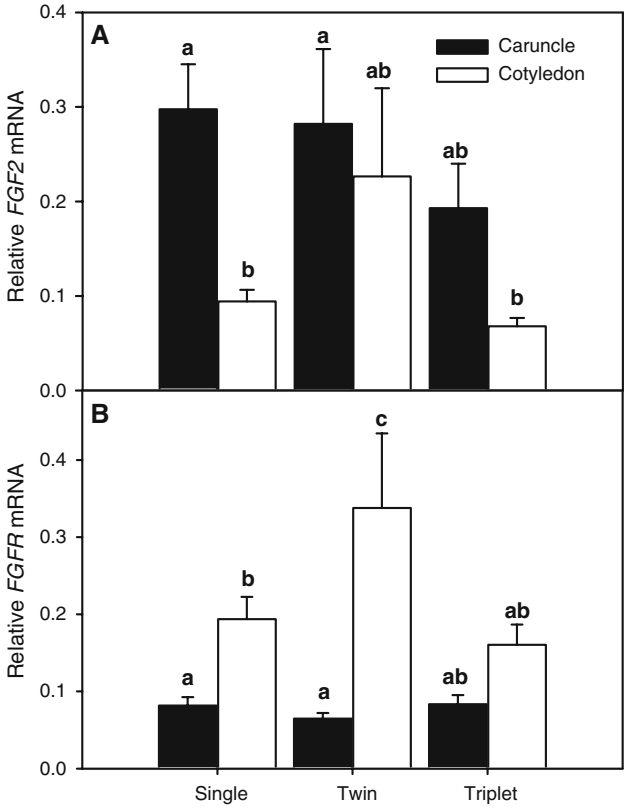

Fig. 3 Caruncular and cotyledonary mRNA expression of *FGF2* (**a**) and *FGFR* (**b**) from singleton, twin and triplet pregnancies on day 140 of gestation. [a,b]Means ± SEM within a tissue differ, $P < 0.05$

increased fetal numbers at day 100 of gestation, while placental weights by day 70 in twin and triplet pregnancies are already reduced compared to singleton pregnancies [39]. Furthermore, placental efficiency in singleton compared to multiple pregnancies was not different, indicating that the relationship between fetal weight to placental weight was similar in singleton and multiple pregnancies. In contrast, Dwyer et al. [7] demonstrated that placental efficiencies were increased in triplet pregnancies compared to singleton and twin pregnancies at birth in sheep. Furthermore, pigs selected for an increased litter size had piglets with increased placental efficiencies [25, 26]. Potentially, these discrepancies could be explained by differing breeds of sheep and species compared to this study, as well as the low number of ewes carrying triplets.

Vonnahme and Ford [25] further demonstrated that placental vascularity from pigs selected for increased placental efficiencies and from pigs selected for low placental efficiencies was similar. While placental vascularity did not differ, mRNA for *Vegf* and *Vegfr1* was increased in the fetal placentas in the high placental efficiency group compared to the low placental efficiency group [25]. The authors indicated that VEGF may be acting as a vascular permeability factor, and therefore, an increase in placental nutrient delivery may be occurring. Similarly, in this experiment, while we observed no differences in capillary vascularity, cotyledonary mRNA for *Vegf*, *Vegfr1*, and *Vegfr2* were increased in twin pregnancies compared to singleton pregnancies, potentially acting to increase vascular permeability. However, *Vegf*, *Vegfr1*, and *Vegfr2* mRNA expression in placentomes was similar for triplets and singleton pregnancies in this study. Moreover, VEGF protein expression within the placentome decreased with increased numbers of fetuses. While all ewes in the current study were slaughtered at a similar time point relative to breeding, it is known that gestation length in ewes carrying multiples is reduced compared to singleton [39]. This may have impacted the discrepancies between the mRNA and protein expression of angiogenic factors investigated in this study. Interestingly, the localization of these factors is different between maternal and fetal tissues within the

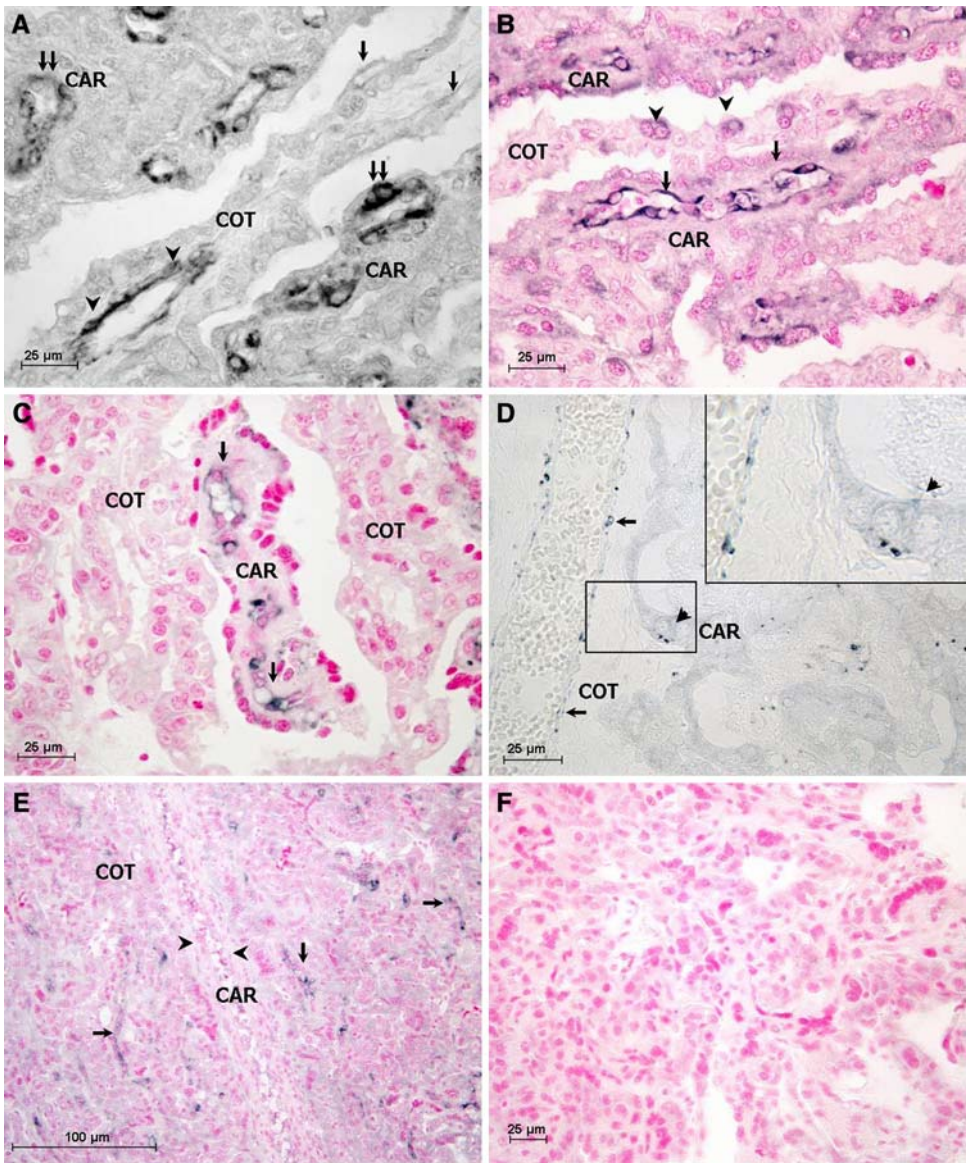

Fig. 4 Representative photomicrograph of (**a**) VEGF, (**b**) VEGFR2, (**c**, **e**) FGF2, and (**d**) FGFR, immunohistochemical staining in the caruncle (CAR) and cotyledon (COT). VEGF in COT (**a**) was localized in the major blood vessels that were greater than 15 µm in diameter (arrowheads), and was not present in capillaries (arrows). In CAR, VEGF was localized in the walls of most of the blood vessels (double arrows). Staining for VEGFR2 in COT (**b**) is localized only in the binucleate cells (arrowheads). In CAR, VEGFR2 was localized in walls of many of the maternal blood vessels (arrows). (**c**) demonstrates localization of FGF2 in CAR (arrows) blood vessels. (**d**) demonstrates scattered, punctuate, positive staining for FGFR in CAR and COT. In COT, the majority of FGFR staining was localized in the walls of the major arteries (arrows) and binucleate cells (arrowheads). Insert shows greater magnification of marked area with binucleate cell (arrowhead). (**e**) Pillar of maternal CAR tissue with a main supplying artery of diameter ~25 µm (arrowheads) shows no FGF2 staining. Maternal capillaries of ~10 µm (arrows), however show positive staining for FGF2. Size bar indicates 25 µm (**a**, **b**, **c**, **d**) or 100 µm (**e**). (**f**) Control staining with normal mouse or rabbit serum in place of primary antibody is shown

placentome. While VEGF was localized in all sizes of maternal blood vessels, VEGF was expressed only in fetal arterioles. Furthermore, Zheng and coworkers [27] reported VEGF localized to placental endothelial cells. Decreases in VEGF may impact NO production necessary for vasodilation [40], causing a stress and initiating the parturient process earlier in multiple pregnancies compared to singleton pregnancies [39]. However, this subject requires further investigation.

Vatnik et al. [41] indicated that in twin pregnancies where one fetus was removed surgically on day 115, there is an increase of the placental size of the remaining fetus on day 136. Furthermore, the remaining twin was similar in weight to singleton fetuses, which were both heavier than

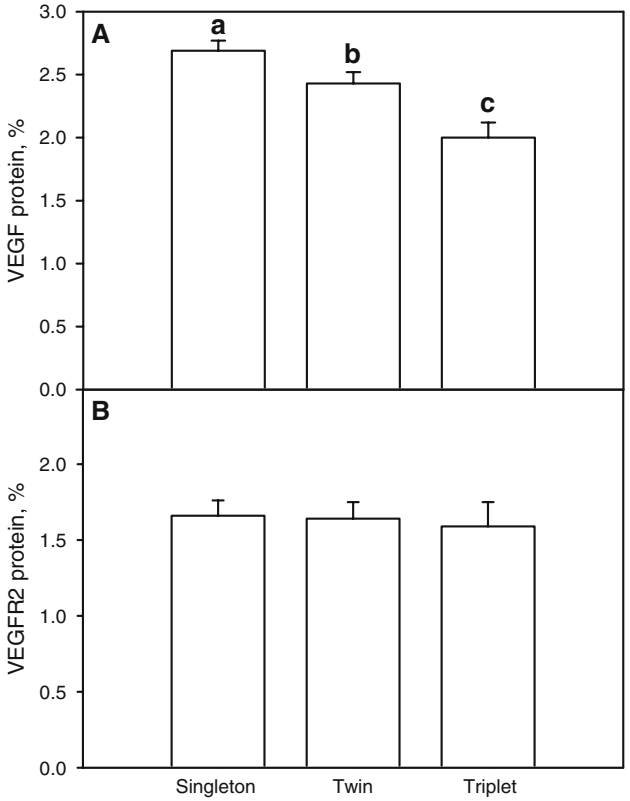

Fig. 5 Placentome VEGF (**a**) and VEGFR2 (**b**) protein expression as determined by immunohistochemistry from singleton, twin and triplet pregnancies on day 140 of gestation. [a,b]Means ± SEM differ, $P < 0.01$

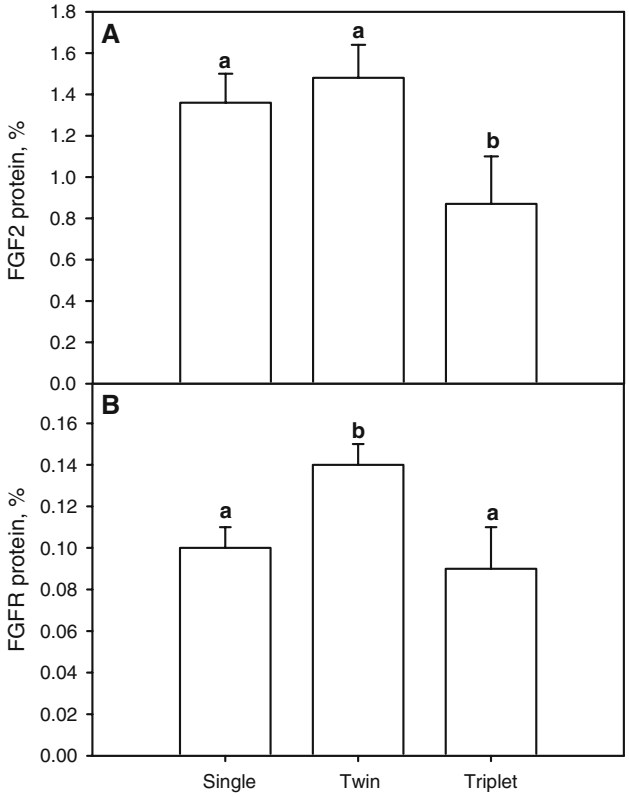

Fig. 6 Placentome FGF2 (**a**) and FGFR (**b**) protein expression as determined by immunohistochemistry from singleton, twin and triplet pregnancies on day 140 of gestation. Overall ANOVA for FGF2 had a *P*-value = 0.10 with unprotected means separation had a P-value of singleton and twin (**a**) versus triplet (**b**) = 0.05. For FGFR: [a,b]Means ± SEM within a tissue differ, $P = 0.07$

individual twin weights. This study demonstrates the plasticity of placental growth and metabolism in response to the loss of a fetus [41]. The increase in placental size must have allowed for an increase in nutrient delivery, either by increasing total capillary vascularity, increasing blood flow, or both, although these measurements were not obtained in that study. While we did have some conceptus loss in the current study (see Materials and Methods), these losses occurred before day 45 to 60 of gestation, as no fetal or placental evidence remained at the time of ultrasound. In light of no late fetal loss (after ~45 days of gestation), the capillary area density, capillary number density, and capillary surface area density did not differ among singleton, twin or triplet on day 140 of pregnancy. Further, the placental efficiency measurements did not differ, indicating no compensation from the remaining conceptuses. Therefore, monitoring uteroplacental blood flow continuously in ewes would allow the determination of how fetal numbers, fetal losses, and stage of pregnancy impacts blood flow.

In order to maintain normal oxygen and nutrient delivery for proper fetal growth, uteroplacental blood flow increases during pregnancy [3, 18, 42]. The uterine vasculature must grow, develop, and vasodilate in order to support this increase in blood flow. On day 105 of pregnancy, uterine blood flow is ~50% higher in ewes carrying triplets compared to singletons or twins [2]. This increase may indeed be driven by VEGF enhancing NO production [40] and therefore vasodilation in the triplet pregnancies. While NO in circulation increases with increased fetal numbers, VEGF in circulation remains similar in singleton and multiple pregnancies [43]. This difference may be in part to either the secretion rates, or VEGF may be acting locally, and not allowed to enter circulation. As we only determined expression patterns at one time point, the difference between triplet and twin VEGF mRNA and protein expression levels may be the result of VEGF expression relative to the time of parturition. Therefore, the time course of angiogenic factor expression, as well as the rate of NO production, in placentas from singleton, twin and triplet pregnancies in relation to the onset of parturition needs to be investigated.

There was no effect of number of fetuses on *Fgf2* mRNA expression in the current study, but FGF2 protein expression were less in placentomes from triplet pregnancies compared to singleton and twin pregnancies. This

indicates possible posttranslational modifications in FGF2 expression in triplet pregnancies. Both FGFR mRNA and protein expression was increased in twin cotyledonary tissue compared to singleton and triplet pregnancies. While cotyledonary mRNA expression of *Fgf2* increased from day 50 to day 140 of gestation, *Fgf2* mRNA expression remained relatively constant in ovine caruncular tissue [30]. In addition, expression of *Fgf2* mRNA was not correlated with any capillary vascularity measurements in the caruncular tissues but was correlated with cotyledonary capillary vascular growth and increases from day 50 to day 140 in the ewe [30]. Zheng and coworkers [27] demonstrated that both cotyledonary and caruncular tissue from late pregnant ewes secrete FGF2, which may help maintain the vascularity of the placenta later in gestation and regulate tissue remodeling to prepare for parturition. As mentioned above, ewes carrying multiple fetuses tend to have shorter gestation lengths [39] and if parturition were eminent for the triplets in this study, FGF2 protein expression may have been altered at the time of tissue collection. The mRNA of *Fgfr3*, the specific FGFR investigated in this study, is expressed in the human placenta during the first two trimesters, with mRNA expression being undetectable in the third trimester [44]. While we cannot comment on the change of placental FGFR3 throughout pregnancy in singleton versus multiple fetuses, the number of fetuses may alter the timing of expression in the fibroblast growth factor system; therefore, additional studies should examine the expression and role of members of FGF system throughout pregnancy.

Size of the placenta is an important consideration when determining the potential nutrient uptake of the fetus. For the same animals as used in this study, Grazul-Bilska et al. [6] reported that the total weight of the placentomes increased with increased number of fetuses. The numbers of total placentomes was greater in ewes carrying twins and triplets than in ewes carrying singletons. However, the number of placentomes per fetus was reduced with increasing fetal numbers (i.e., 67.0, 43.5, and 28.5 $\pm$ 4.3 placentomes per fetus in singleton, twin, and triplet pregnancies, respectively; [6]). Furthermore, while placentome weight per fetus was reduced in twin and triplet pregnancies compared with singleton pregnancies, the mean weight of an individual placentome in the triplet pregnancy was $\sim$130% greater than placentomes from twin and singleton pregnancies [6]. With greater competition for maternal nutrients with less placentomes per fetus in multiple pregnancies, different mechanisms to obtain these nutrients seem plausible. It is possible that while angiogenic factor expression is increased in twin pregnancies, the size of individual placentomes is increased in triplet pregnancies. It appears that these two different methods of extracting nutrients (i.e., nutrient exchange versus size) may have the same outcome as there was no difference in average fetal weight between twin and triplet pregnancies in this study [6]. However, fetuses from both twin and triplet pregnancies were lighter compared to singletons. While we have calculated the total capillary volume in singleton versus multiple pregnancies, investigating functional capillary activity (i.e., nutrient flux) is warranted.

In summary, it appears that while there is no difference in capillary vascularity of placentomes from singleton, twin or triplet pregnancies in ewes, angiogenic factor expression is altered. Decreased birth weights which have been shown to increase the risk for metabolic and coronary diseases later in life may be prevented if the timing and levels of angiogenic factors are understood [17, 45]. It is now becoming evident that reduced fetal weight is due to a decrease in uterine and/or umbilical blood flows in several sheep models including nutrient restriction in adult ewes, overnutrition in peripubertal ewes, heat stress, and hypobaric ewes (for review see [23]). Factors, such as nitric oxide, that can be used to regulate blood flow in multiple pregnancies needs to be investigated.

Methods

Animal procedures

All procedures were performed during the normal breeding season (September–February) at the Animal Nutrition and Physiology Center of North Dakota State University (NDSU) located in Fargo, ND, USA (46.9° N, 96.8° W), and were approved (#A0034) by the Institutional Animal Care and Use Committee of NDSU. Tissues processed and evaluated in this study were obtained in the previous study described in detail by Grazul-Bilska et al. [6]. Briefly, all ewes used for this study were from the same flock, and were white-faced, predominantly Targhee and Rambouillet, crossbred ewes that exhibited a spontaneous estrous cycle of normal duration (15–17 days). Ewes were checked for estrus twice daily by using vasectomized rams. Multiple pregnancies were established by transferring 2 or 3 embryos from crossbred donor ewes on day 5 after in vitro fertilization (IVF; [6]) to crossbred recipients on day 5 after onset of estrus. Recipients were synchronized by prostaglandin treatment as previously described [6]. Pregnancy was confirmed in recipient ewes using ultrasonography (Classic Ultrasound Equipment Ltd, Tequesta, FL, USA) between day 45 and 60 after embryo transfer. Of 18 detected pregnancies, 16 pregnancies were established after transfer of three embryos/recipient, and two pregnancies were established after transfer of two embryos/recipient. Placentomes were collected from ewes carrying singletons ($n = 8$), twin ($n = 7$) and triplet ($n = 3$) fetuses on day

140 of gestation (normal length of gestation ~145 days). After removal of the fetus(es), placentomes were fixed in Carnoy's solution (see below) to determine vascularity in caruncular and cotyledonary tissue [30]. Only type A placentomes, as characterized by Vatnick et al. [41] were used in this experiment. Cotyledonary tissue from several placentomes was then manually removed from the caruncular tissue, and snap frozen for RNA extraction.

Tissue perfusion, histochemical staining and image analysis

Placentomes were perfused as previously described [30]. Briefly, caruncular and cotyledonary tissues were fixed with Carnoy's solution by perfusion of the main arterial vessel, followed by a vascular casting resin (Mercox, Ladd Industries, Burlington, VT). Then tissues were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and periodic acid–Schiff's reagent using previously reported procedures [46]. Photomicrographs were taken for 12 randomly chosen areas per tissue per sheep to determine vascularity by image analysis (Image-Pro Plus 5.0; Media Cybernetics, Silver Spring, MD; [30]). The following parameters were determined for caruncular and cotyledonary tissue in each section: capillary number density (number of capillaries per unit area tissue), capillary cross sectional area density (capillary area per unit tissue area), capillary surface density (capillary circumference per unit area tissue), and average cross sectional area per capillary. The data for capillary parameters measured were averaged across the 12 images for each sheep prior to statistical analysis.

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Expression of mRNA levels for *Vegf*, *Vegfr1*, *Vegfr2*, *Fgf2*, and *Fgfr* were determined using quantitative real-time RT-PCR as previously described [47, 48]. Quality and quantity of total RNA were determined via capillary electrophoresis using an Agilent 2100 Bioanalyzer (Agilent Technologies, Wilminton, DE). All reagents for the real-time RT-PCR were purchased from Applied Biosystems (Foster City, CA).

Immunohistochemistry

Detection of factor VIII, VEGF, VEGFR1, FGF2, and FGFR protein was performed as previously described [49–51]. Briefly, sections of placentomes fixed in formalin or Carnoy's solution were rinsed several times in PBS containing Triton-X100 (0.3%, v/v) and then were treated for 20 min with blocking buffer [PBS containing normal goat serum (1–2%, v/v)] followed by incubation with specific primary antibody overnight at 4°C. Carnoy's fixed tissues were utilized for factor VIII (Sigma–Aldrich, St. Louis, MO) and VEGF [51] antibodies. Formalin fixed tissues were utilized for VEGFR, FGF2, and FGFR antibodies (Santa Cruz Biotechnology Research, Santa Cruz, CA, for VEGFR and FGF2; Zymed, San Francisco, CA, for FGFR). Dilution for all primary antibodies was 1:100. Primary antibody was detected by using a secondary antibody (horse anti-rabbit with horseradish peroxidase for factor VIII, VEGF, VEGFR, and FGF2; goat anti-mouse IgM biotinylated for FGFR) and a streptavidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA; The Impress kit for factor VIII, VEGF, VEGFR, and FGF2; and the Elite ABC kit for FGFR). Control sections were incubated with normal mouse or rabbit serum in place of primary antibody and did not express any positive staining. After immunohistochemical detection of FGF2 and VEGFR1, tissue sections were counterstained with nuclear fast red.

Image analysis

All images were taken from areas with relatively uniform morphology, and areas with connective tissue tracts were avoided. In order to determine relative protein expression of specific antigens, the percentage of the total area that exhibited positive staining was evaluated quantitatively with an image analysis system (Image Pro-Plus, Media Cybernetics, Silver Spring, MD) as described previously [48]. For each placentome, 8–10 randomly chosen fields (0.025 mm^2 per field) were evaluated in each tissue section ($n = 8$–10 measurements/placentome). Data are expressed as mean percentage ± SEM of the total area that exhibited positive staining within each field.

Statistical analysis

Placental efficiency was calculated by dividing total fetal weight by the placentome weight. Fetal and placental weights were previously published [6]. Data were analyzed using the Proc GLM procedures of SAS (SAS, 1999). Class statements included tissue and number of fetuses. Model statement included placental efficiency, cotyledonary and caruncular capillary area density, capillary number density, capillary surface density, area per capillary, placentome factor VIII, VEGF, VEGFR1, FGF2, and FGFR protein expression, and cotyledonary and caruncular *Vegf*, *Vegfr1*, *Vegfr2*, *Fgf2*, and *Fgfr* mRNA expression. Means were separated using the LSMEANS procedures of SAS. Effects were considered significant when $P \leq 0.05$ and as a trend/tendency between $P = 0.06$ and 0.10 unless otherwise stated.

Acknowledgments The authors would like to thank Dr. Jerzy J. Bilski, Mr. James D. Kirsch, Mr. Kim C. Kraft, Dr. Chainarong Navanukraw, and Mr. Robert Weigl for their assistance in animal handling and data collection. This work was supported, in part, by NIH grant HL64141 to LPR and DAR, and ND SBARE grant to ATG-B, LPR and DAR and by NIH grant number P20 RR016741 from the INBRE program of the National Center for Research Resources.

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