Veratrum-Induced Placental Dysplasia in Sheep

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Abstract

Cyclopamine, a steroidal alkaloid from *Veratrum californicum*, is teratogenic causing a range of birth defects including cyclopia (synophthalmia) as well as other craniofacial and structural malformations. Previous studies have indicated that fetuses with cyclopia are smaller, under developed, and appear premature compared to gestational matched normal fetuses. Preliminary observations suggest this could be due to placental dysplasia. The objective of this study was to determine if there are placental dysplasias in ewes with fetuses with synophthalmia and other less severe craniofacial malformations. Ewes were dosed orally twice on gestation day (GD) 14 with 0.88 g/kg of dried *V. californicum*. Pregnancy, pre-partum fetal malformations, and placentome diameter were determined by ultrasound imaging on GD 45, 60, 75, 105, and 135. At GD 135 the ewes were euthanized and the fetuses were assessed for gross malformations and several fetal measurements were made as well as several measurements in the ewe. There was no difference between the controls and the treated animals in the placentome diameter measurements made in utero by ultrasound imaging. The 23 treated ewes were carrying 26 fetuses. Eleven of the fetuses were cyclopic, nine had other craniofacial malformations including maxillary dysplasia, mandibular micrognathia, and superior deviation of the rostral mandible, while six fetuses appeared normal. Cyclopic fetuses were smaller than fetuses with less severe craniofacial malformations, which were similar in size to normal fetuses. The number of placentomes, placentome area, and placentome weight were all significantly smaller for ewes with cyclopic fetuses. There was no difference in the size or weight of the fetal pituitary glands between the different groups of fetuses. However, weights of the fetal adrenal glands were significantly less in the cyclopic fetuses. In summary, there appears to be a correlation between the severity of the malformed fetuses and placental dysplasia.

Abbreviation

GD, Gestational Day

Keywords: cyclopamine, cyclopia, holoprosencephaly, sheep, synophthalmia, *Veratrum californicum*

Introduction

Cyclopia and a number of other teratogenic malformations occur in lambs when pregnant ewes graze *Veratrum californicum* early in gestation (Binns et al. 1962, Binns et al. 1965, Keeler et al. 1985, Keeler and Stuart 1987, Keeler 1990). Incidences as high as 25 percent were reported in flocks of 5,000-10,000 range ewes (Binns et al. 1963). Early evaluation of the chronology of teratogenicity of *V. californicum* in sheep indicated that gestation day (GD) 14 is the critical day for synophthalmia malformations to occur (Binns et al. 1965). Early embryonic death and resorption were

later associated with low reproductive rates when sheep were grazed in areas with abundant *V. californicum* (Binns et al. 1963, Van Kampen et al. 1969). The alkaloids responsible for terata induction in *V. californicum* have been identified as jervine, 11-deoxojervine (renamed cyclopamine), and cycloposine (the glycoside of cyclopamine) (Keeler and Binns 1968). The mechanism of cyclopamine-induced birth defects has been shown to result from the inhibition of the Sonic Hedgehog signal transduction pathway (Cooper et al. 1998, Incardona et al. 1998). The Hedgehog signaling pathway plays an integral role in cell growth and differentiation, including embryonic development of the eyes and maxilla (Rubenstein and Beachy 1998, Lum and Beachy 2004).

Previous studies demonstrated that fetuses with cyclopia were smaller, under developed, and appeared premature compared to normal fetuses of similar gestation age (Welch et al. 2009). Preliminary observations suggested this could be due to placental dysplasia (unpublished observations). The objective of this study was to determine if there are placental dysplasias in ewes with fetuses with synophthalmia and other less severe craniofacial malformations.

Materials and Methods

Plant Material

Root material from *V. californicum* plants was used as the source of cyclopamine for the oral dosing experiments in this study. It has been demonstrated that both the aerial and root/rhizome portions of the plant contain the teratogen cyclopamine (Keeler and Binns 1966a), and that both can induce "monkey faced lamb" defects (Binns et al. 1965). However, the concentration of cyclopamine is 5-10 times higher in root material (Keeler and Binns 1966a, 1966b, 1971). The plant material was collected in Muldoon Canyon at the headwaters of the Lost River Drainage in Idaho. Plant material was transported to our laboratory, air dried in sunlight, finely chopped, and stored in an enclosed shed at ambient temperature. Extraction of *V. californicum* for cyclopamine analysis was accomplished as described previously (Welch et al. 2009).

Animal Studies

Twenty-seven western white-faced ewes weighing 78 ± 11 kg were synchronized in estrus using intravaginal sponges impregnated with fluorogestone acetate (Intervet International B.V., Netherlands). Each ewe was hand mated to Suffolk rams 3 times a day for 3 days following removal of the intravaginal sponges; the last day that each ewe exhibited standing estrus was considered day 0 of gestation (Keeler and Stuart 1987, Keeler and Baker 1989, Jainudeen et al. 2000). Each ewe was dosed at 7 a.m. and 3 p.m. on GD 14 with ground plant material in order to limit maternal toxic effects of *V. californicum*. Twenty-three ewes were dosed with 0.88 g *V. californicum*/kg BW, and four ewes were dosed with 0.88 g alfalfa/kg BW as controls.

Ewes were evaluated via ultrasound imaging for pregnancy on GD 30. Pregnancy, pre-partum fetal malformations, and placentome diameter were determined by ultrasound imaging on GD 45, 60, 75, 105, and 135. The ewes were examined transabdominally using an Aloka SSD-900V scanner fitted with a 5 MHz convex electronic transducer (Wallingford, CT). The ewes were restrained on their backs to facilitate access to the hairless areas of the abdominal wall just in front of the udder. All ewes, including controls, were euthanized on GD 135, and the fetuses were assessed for gross malformations, and several fetal measurements were made as well as several placental measurements as listed in tables 2-5. All uterine and placental measurements are reported once for each ewe for each comparison and not for each fetus. Normal gestation for these sheep is approximately 150 days.

Analysis and Statistics

Statistical comparisons between two groups were performed using a Student's *T*-test and between three or more groups using ANOVA with a Bonferroni post hoc test of significance between individual groups as pairwise comparisons. Differences were considered significant at $P < 0.05$.

Results

Of the four ewes treated with alfalfa on GD 14, one was found to not be pregnant on GD 30 (table 1). The remaining three ewes each had normal fetuses (five fetuses total) at GD 135. Twenty-three ewes were treated with *V. californicum* on GD 14. Three of the 23 ewes were not pregnant on GD 30 (table 1). Five of the remaining 20 treated ewes were not pregnant on GD 135, indicating that embryonic/fetal death had occurred in these ewes. Twenty-six fetuses were found in the remaining 15 ewes. Eleven of those fetuses had cyclopia (synophthalmia), nine had other less severe craniofacial malformations (which are referred to as monkey-faced fetuses), and six were normal. In this study, we use the term "cyclopia" to refer to fetuses with a single eye

Table 1. Summary of the effects of *Veratrum* treatment on embryonic loss, birth defects, and number of fetuses

Category	Number of ewes	Number of fetuses
Breeding results		
Control ewes bred	4	
Control ewes not pregnant on GD 30	1	
Control ewes pregnant on GD 135	3	5
Treated ewes bred	23	
Treated ewes not pregnant on GD 30	3	
Treated ewes pregnant on GD 135	15	26
Treated ewes with confirmed loss of embryo after GD 30	5	
Type of birth defect		
Cyclops fetus	8	11
Monkey-faced fetus	6	9
Normal fetus	7	11
Normal fetus from treated ewe	4	6
Normal fetus from control ewe	3	5
Mix of normal & monkey-faced fetus	1	2
Mix of cyclops & monkey-faced fetus	2	4
Number of fetuses		
Single	7	7
Normal		2
Monkey-faced fetus		1
Cyclops fetus		4
Twins	9	18
Normal		9
Monkey-faced fetus		5
Cyclops fetus		4
Triplets	2	6
Monkey-faced fetus		3
Cyclops fetus		3

Figure 1. Craniofacial defects in fetuses associated with maternal ingestion of *V. californicum.*

socket. All other fetuses with less severe craniofacial malformations that have two eye sockets are referred to as monkey-faced fetuses. Various representations of the craniofacial malformations are represented in figure 1.

Seven ewes had single fetuses, two normal fetuses (one from a treated ewe and one from a control ewe), one monkey-faced fetus, and four cyclopic fetuses (table 1). Nine ewes had twin fetuses, nine normal fetuses (four from control ewes and five from treated ewes), five monkey-faced fetuses, and four cyclopic fetuses. Two ewes had triplet fetuses, three monkey-faced fetuses and three cyclopic fetuses. Each set of triplet fetuses were the same, i.e., all cyclopic or all monkey-faced. However, three of the sets of twins were mixed, one set of normal and monkey-faced fetuses and two sets of cyclopic and monkey-faced fetuses.

Ultrasound imaging was used to measure the in utero placentome diameter on GD 45, 60, 75, 105, and 135 (figure 2). Measurements were recorded for each ewe throughout the experiment. Once the experiment was over, the ewes were then categorized into either treated or control, depending upon their treatment. The ewes were also categorized according to the malformations that their fetuses had, i.e., normal, monkey-faced, or cyclopic. A statistical comparison of the in utero placentome diameters demonstrated no difference between control and treated ewes ($P = 0.16$, figure 2A), and there was no difference amongst three outcome groups ($P = 0.19$, figure 2B). In both comparisons, there was a day effect ($P < 0.001$), as the placentomes were shown to increase in size from GD 45 to GD 60. However, there was no group x day effect for either comparison ($P = 0.27$ and $P = 0.41$).

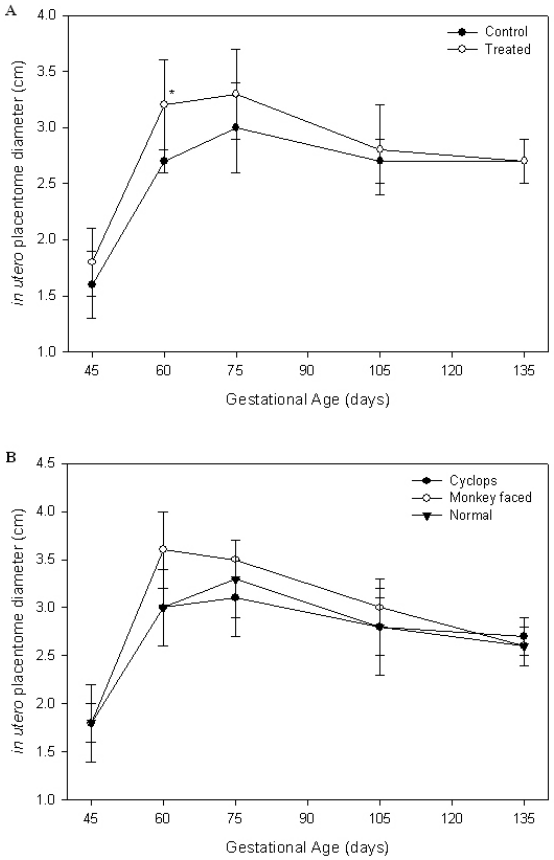

Figure 2. Diameter of placentomes measured *in utero* via ultrasound imaging. Results represent the mean ± SD of 3 to 15 ewes per group. (A) Control versus treated ewes; (B) ewes with cyclops, monkey-faced, or normal lambs. * $P < 0.05$ as compared to controls.

At GD 135 all ewes were euthanized and their gravid uterus was removed. Numerous maternal and fetal measurements were made in order to determine if either *Veratrum* treatment or the type of fetus was correlated with the development of placentation. Due to the complexity and potential confounding factors, four different comparisons were performed: (1) comparison of treatment, (2) comparison of the type of fetuses, (3) comparison of the type of twin fetuses, and (4) comparison of the number of fetuses. First, the measurements from normal fetuses from treated ewes were compared to the measurements from the normal fetuses from control ewes to determine if *Veratrum* treatment had any effect (table 2). The only difference observed was that the fetuses from treated ewes had significantly larger adrenal glands as measured by weight ($P = 0.001$). Therefore, for the remaining comparisons, the normal fetus data for the treated and control ewes were combined in order to increase the sample size for statistical power.

The comparison of the measurements between the types of fetus demonstrated that there was a difference in the number of placentomes and the placentome weight between ewes with normal and cyclopic fetuses, with ewes with normal fetuses having more placentomes that were also larger (table 3). The cyclopic fetuses were found to be significantly smaller than both the monkey-faced and normal fetuses, demonstrated by the lower fetal weight and smaller crown to rump length, abdominal and thoracic circumference, as well as smaller fetal brain and adrenal weights. Of note, a comparison of the cyclopic fetus fetal adrenal weights compared to only the fetal adrenal weight from fetuses born to control ewes is still statistically significant ($P < 0.001$). Similarly, the monkey-faced fetuses were also found to be smaller than the normal fetuses. In general, similar results were observed when restricting the comparison of the types of fetuses to only fetuses that were part of a set of twins (table 4). Notable exceptions were the fact that the gravid uterus weight for ewes with cyclopic fetuses was less than that of ewes with normal fetuses. Even though ewes with twin normal fetuses had more placentomes than ewes with twin cyclopic fetuses, there was no difference in the average placentome weights.

A comparison of the measurements when categorized according to the number of fetuses from each ewe found only differences that would be expected with increasing number of fetuses in each gravid uterus, i.e., the gravid uterus weight, empty uterus weight, as well as the allantoic and amniotic fluid volumes were larger in ewes with multiple fetuses (table 5). The one additional difference observed was single fetuses had smaller average placentome weights than twin fetuses.

Discussion

Early embryonic development and maternal recognition of pregnancy require numerous processes to occur at specific times as well as specific hormonal balances in the uterine environment (DeSesso 2006). This environment is created by both the embryo and the uterus (Ashworth and Bazer 1989). The importance of environment and timing is highlighted by the fact that approximately 30 percent of the embryos are lost during early development (Dixon et al. 2007). The uterus exerts its own influence on embryonic development through histotrophic nutrition (Bazer et

Table 2. A comparison of normal fetuses from control ewes and normal fetuses from treated ewes

Measurement	Treated ewes			Control ewes		
	AVG	SD	n	AVG	SD	n
Gravid uterus weight (kg)	14.6	± 3.2	4	12.0	± 3.2	3
Empty uterus weight (kg)	2.5	± 0.4	4	2.3	± 0.3	3
Allantoic & amniotic fluid volume (l)	2.3	± 0.8	4	1.8	± 0.7	3
Number of placentomes	83.0	± 17.7	4	91.7	± 3.1	3
Placentome area (cm^2)	13.3	± 3.4	4	7.9	± 1.3	3
Placentome weight (g)	14.8	± 1.6	4	12.5	± 2.7	3
Fetal weight (kg)	5.6	± 0.8	6	4.9	± 0.4	5
Crown rump length (cm)	51.6	± 4.0	6	50.5	± 4.7	5
Abdominal circumference (cm)	33.2	± 2.6	6	35.7	± 2.2	5
Thoracic circumference (cm)	35.8	± 1.7	6	35.9	± 1.3	5
Fetal brain weight (g)	56.9	± 4.8	6	53.6	± 3.6	5
Fetal pituitary weight (g)	0.5	± 0.3	6	0.2	± 0.0	5
Fetal adrenal weight (g)	0.6	± 0.1*	6	0.4	± 0.1	5
Fetal trachea diameter (cm)	1.1	± 0.5	6	0.7	± 0.1	5

* = different from control at $P < 0.05$

Table 3. Comparison of measurements between fetuses with different defects

Measurement	Cyclops fetuses		Monkey-faced fetuses		Normal fetuses	
	AVG±SD	n	AVG±SD	n	AVG±SD	n
Gravid uterus weight (kg)	9.2 ± 2.8	8	13.9 ± 5.8	6	13.5 ± 3.2	7
Empty uterus weight (kg)	1.8 ± 0.5	8	2.2 ± 0.7	6	2.4 ± 0.4	7
Allantoic & amnionic fluid volume (l)	3.4 ± 1.2	8	3.5 ± 1.4	6	2.1 ± 0.7	7
Number of placentomes	59.1 ± 21.6 [n]	8	76.5 ± 22.0	6	86.7 ± 13.5 [c]	7
Placentome area (cm^2)	7.7 ± 1.4	8	8.8 ± 1.8	6	10.9 ± 3.8	7
Placentome weight (g)	9.5 ± 2.5 [n]	8	12.0 ± 3.2	6	13.8 ± 2.3 [c]	7
Fetal weight (kg)	2.3 ± 0.5 [m,n]	11	4.2 ± 0.9 [c,n]	9	5.3 ± 0.7 [c,m]	11
Crown rump length (cm)	41.3 ± 4.1 [m,n]	11	49.0 ± 3.6 [c]	8	51.1 ± 4.2 [c]	11
Abdominal circumference (cm)	27.1 ± 2.6 [n]	11	28.4 ± 2.5 [n]	8	34.3 ± 2.6 [c,m]	11
Thoracic circumference (cm)	27.0 ± 1.4 [m,n]	11	30.9 ± 2.2 [c,n]	8	35.9 ± 1.5 [c,m]	11
Fetal brain weight (g)	13.5 ± 8.6 [m,n]	11	33.5 ± 10.4 [c,n]	8	54.2 ± 4.7 [c,m]	11
Fetal pituitary weight (g)	0.3 ± 0.2	11	0.2 ± 0.2	7	0.3 ± 0.3	11
Fetal adrenal weight (g)	0.2 ± 0.1 [m,n]	11	1.5 ± 0.3 [c]	9	0.6 ± 0.1 [c]	11
Fetal trachea diameter (cm)	0.5 ± 0.3	11	0.9 ± 0.5	9	0.9 ± 0.4	11

[c] = different from cyclops fetuses at $P < 0.05$
[m] = different from monkey-faced fetuses at $P < 0.05$
[n] = different from normal fetuses at $P < 0.05$

Table 4. Comparison of measurements between twin fetuses with different defects

Measurement	Cyclops fetuses		Monkey-faced fetuses		Normal fetuses	
	AVG±SD	n	AVG±SD	n	AVG±SD	n
Gravid uterus weight (kg)	11.0 ± 1.1 [n]	3	13.0 ± 2.8	4	15.2 ± 1.3 [c]	5
Empty uterus weight (kg)	2.0 ± 0.4	3	2.2 ± 0.5	4	2.6 ± 0.2	5
Allantoic & amnionic fluid volume (l)	3.2 ± 0.8	3	2.7 ± 0.6	4	2.5 ± 0.5	5
Number of placentomes	56.0 ± 15.7 [n]	3	77.3 ± 23.7	4	91.8 ± 3.8 [c]	5
Placentome area (cm^2)	9.1 ± 0.3	3	8.9 ± 0.5	4	10.9 ± 3.7	5
Placentome weight (g)	12.3 ± 0.3	3	12.5 ± 1.4	4	14.5 ± 2.3	5
Fetal weight (kg)	2.6 ± 0.4 [m,n]	4	4.4 ± 0.7 [c]	5	5.1 ± 0.7 [c]	9
Crown rump length (cm)	44.1 ± 5.0 [m,n]	4	50.1 ± 1.6 [c]	4	49.7 ± 2.9 [c]	9
Abdominal circumference (cm)	25.6 ± 2.9 [n]	4	28.7 ± 3.6 [n]	4	34.0 ± 2.6 [c,m]	9
Thoracic circumference (cm)	27.5 ± 1.4 [m,n]	4	31.1 ± 2.2 [c,n]	4	35.7 ± 1.5 [c,m]	9
Fetal brain weight (g)	21.3 ± 3.2 [m,n]	4	35.2 ± 11.7 [c,n]	5	54.5 ± 5.0 [c,m]	9
Fetal pituitary weight (g)	0.3 ± 0.1	3	0.3 ± 0.1	4	0.3 ± 0.3	9
Fetal adrenal weight (g)	0.2 ± 0.1 [n]	4	0.4 ± 0.2	5	0.6 ± 0.1 [c]	9
Fetal trachea diameter (cm)	0.8 ± 0.4	4	0.8 ± 0.5	5	0.9 ± 0.4	9

[c] = different from cyclops fetuses at $P < 0.05$
[m] = different from monkey-faced fetuses at $P < 0.05$
[n] = different from normal fetuses at $P < 0.05$

Table 5. Comparison of measurements between single, twin, and triplet fetuses

Measurement	Single fetus		Twin fetuses		Triplet fetuses	
	AVG±SD	n	AVG±SD	n	AVG±SD	n
Gravid uterus weight (kg)	7.6 ± 1.6 [2,3]	7	13.8 ± 2.4 [1]	9	15.2 ± 7.6 [1]	2
Empty uterus weight (kg)	1.6 ± 0.4 [2,3]	7	2.4 ± 0.4 [1]	9	2.6 ± 0.3 [1]	2
Allantoic & amnionic fluid volume (l)	2.8 ± 1.5 [3]	7	2.7 ± 0.7 [3]	9	2.5 ± 0.9 [1,2]	2
Number of placentomes	58.6 ± 19.0	7	80.1 ± 19.9	9	91.8 ± 3.5	2
Placentome area (cm^2)	7.9 ± 3.4	7	10.0 ± 2.8	9	10.9 ± 2.8	2
Placentome weight (g)	8.5 ± 2.8 [2]	7	13.4 ± 1.8 [1]	9	14.5 ± 4.0	2
Fetal weight (kg)	3.5 ± 1.7	7	4.3 ± 1.2	18	5.1 ± 1.7	6
Crown rump length (cm)	45.7 ± 8.5	7	48.4 ± 3.9	17	49.7 ± 2.9	6
Abdominal circumference (cm)	30.4 ± 4.1	7	30.8 ± 4.6	17	34.0 ± 2.6	6
Thoracic circumference (cm)	30.3 ± 4.6	7	32.7 ± 3.8	17	35.7 ± 1.5	6
Fetal brain weight (g)	22.3 ± 22.2	7	41.8 ± 15.6	18	54.5 ± 5.0	5
Fetal pituitary weight (g)	0.3 ± 0.3	4	0.3 ± 0.2	16	0.3 ± 0.3	4
Fetal adrenal weight (g)	0.3 ± 0.2	7	0.4 ± 0.2	18	0.6 ± 0.1	6
Fetal trachea diameter (cm)	0.6 ± 0.4	7	0.8 ± 0.4	18	0.9 ± 0.4	6

[1] = different from single fetuses at $P < 0.05$
[2] = different from twin fetuses at $P < 0.05$
[3] = different from triplet fetuses at $P < 0.05$

al. 1993). In addition to uterine secretions, key embryonic secretions are also required for a synchronous interaction between uterine endometrium and embryonic tissues (Koch et al. 2010). Between GD 8-16 the developing conceptus secretes interferon-τ, which is thought to initiate the process of maternal recognition of pregnancy and is required for normal embryonic development (Spencer et al. 2004). Other studies have identified embryonic proteins associated with early embryonic attachment (Lee et al. 1998). Consequently, any alteration to the uterine environment during the early stages of development of the embryo can affect the ability of the embryo to develop normally, survive, and undergo normal parturition. A number of compounds that are teratogenic, due to their ability to alter the uterine environment or normal embryonic development, are plant toxins (Keeler 1984, Panter et al. 2011). In early studies of *Veratrum*-induced malformations, the observation was made that ewes with severely deformed fetuses had significantly prolonged gestations as a part of the syndrome (Binns et al. 1964, Van Kampen and Ellis 1972). In sheep, parturition is initiated by increased fetal hypothalamic-pituitary-adrenal (HPA) axis activity leading to fetal and maternal prostaglandin production and a rise in the maternal estradiol-progesterone (E2/P4) ratio (Kumarasamy et al. 2005). Estrogen up-regulates the expression of maternal endometrial prostaglandins, which stimulates myometrial contractility and labor ensues (Whittle et al. 2000). Corticotrophin releasing hormone (CRH) can stimulate the fetal release of ACTH to produce a cortisol surge, which leads to the onset of parturition, whereas inhibition of these processes can delay the onset of parturition (Chan et al. 1998). The placenta also plays an important role in normal hormone production, including growth hormone (Handwerger and Freemark 2000). Consequently, it is possible that veratrum treatment is altering normal HPA and/or placental function, resulting in delayed parturition and diminished fetal growth.

The observation was made in a recent study that a set of twin fetuses from a ewe treated with *Veratrum* appeared to be at different stages of development (Welch et al. 2009). A monkey-faced fetus was of normal size and fully covered with wool, similar to normal fetuses. However, its cyclopic twin was approximately two-thirds the size and had no wool. Although it is not uncommon to have normal twin fetuses of differing size, the difference in wool covering of these two twins is very unusual. Interestingly, in the same study, cyclopic fetuses that were surgically removed on GD 200 (normal gestation is 150 days) were of normal size and were fully covered in wool. Taken together, these observations indicate that the development of the cyclopic fetuses is hindered, and therefore more time is required for the fetus to reach normal weight and have normal wool covering. A potential, and possibly related, explanation could be deficiency of the placentation supporting cyclopic fetuses. The observation was made that the placentas from the ewes that delivered at GD 200 were not normal as the placentomes appeared smaller. Unfortunately, no assessments of the placentomes were made from the ewes that had delivered normally around GD 150. Consequently, the objective of this study was to determine if there are placental dysplasias in ewes with fetuses with synophthalmia and other less

severe craniofacial malformations versus ewes with normal fetuses.

There is evidence that placental growth processes during mid-gestation are critical because placental size determined during this period may have important repercussions during late gestation for functional capacity of the placenta to deliver nutrients to the fetus (Mellor 1983, Bell et al. 1987). In normal pregnancy and fetal development, the placental component exhibits a rapid increase in tissue weight until GD 75-80 and then declines (Ehrhardt and Bell 1995). The endometerium and myometrium steadily increase in weight from GD40-100, more than doubling in weight (Ehrhardt and Bell 1995). In this study, we found no difference in the uterine weight from a ewe with a cyclopic fetus versus a ewe with a normal fetus. However, the average placentome weights in ewes with cyclopic fetuses were lower than ewes with normal fetuses. There is a trend for an increase in placentome number from GD 40 to 60 which then remains unchanged after GD 60 (Ehrhardt and Bell 1995). In this study, we found a significantly lower number of placentomes in ewes with cyclopic fetuses versus ewes with normal fetuses. Additionally, using ultrasound imaging, we determined the maximum size of the placentomes to occur around GD 60 for ewes with monkey-faced fetuses and GD 75 for the remaining ewes (figure 2). Our results are similar to those in reported other studies wherein sheep placentas have been shown to display a period of maximal proliferative growth between GD 50-60 and an abrupt cessation of mass accumulation between GD 75-80 (Ehrhardt and Bell 1995). Interestingly, there was no difference in the size (diameter) of the placentomes in ewes with cyclopic fetuses as determined by ultrasound imaging or at necropsy.

In this study, 75 percent of the control ewes were found to be pregnant at GD 30 versus 87 percent of the treated ewes. However, there was not any embryonic, or fetal, loss (as determined by loss of fetus after a positive confirmation on GD 30) in control ewes versus a 25 percent loss in the treated ewes. A recent study using conditional knockout mice demonstrated that the Hedgehog signaling pathway plays an important role in implantation during pregnancy (Harman et al. 2011). In these mice, smoothened, the key Hedgehog pathway signal transducer, was conditionally deleted. Mice with one or two functional smoothened alleles had approximately 9 pups per litter whereas homozygous knockout mice had approximately 4 pups per litter. Interestingly, there was no difference in the average interval between litters or in the percentage of pups that were weaned. There was a significant reduction in the number of implantation sites in knockout mice compared to controls but no difference in the number of corpora lutea. Embryonic loss was not due to insufficient luteal function, as the knockout mice did not differ in serum progesterone concentrations during GD 4-13. The authors suggest that conditional reduction of Hedgehog pathway signaling due to lack of smoothened in the uterus leads to deferred implantation beyond the normal window of receptivity and that delayed implantation was associated with developmental delay in the embryos (Harman et al. 2011).

Normally, the ovine fetus exhibits an exponential growth pattern through GD 40-100. The fetus becomes the largest component of the gravid uterus near GD 100 and continues to gain size throughout the remainder of gestation (Ehrhardt and Bell 1995). In the study with conditional smoothened knockout mice, 20 percent of the fetuses in knockout mice were smaller than the wild type mice (Harman et al. 2011). The cyclopic fetuses assessed in this study were found to be significantly smaller than normal fetuses as determined by fetal weight, crown to rump length, abdominal and thoracic circumference, and fetal brain weight. Particularly striking was the large difference in the fetal brain weights. Several of the cyclopic fetuses had minimal brain tissue in the cranium with the presence of large amounts of fluid (figure 3). Consequently, with such lack of brain development, and likely function, the development of these fetuses would be severely compromised.

Another possible explanation for the delayed development in cyclopic fetuses could be due to defects in the pituitary in the fetuses and subsequently deficiencies in the production of hormones involved in growth and development. In early studies, many severely deformed cyclopic lambs did not have a pituitary gland (Binns et al. 1962). However, in this study, 55 percent of the cyclopic fetuses had a pituitary gland. Additionally, there was no difference in the size of the pituitary glands that were present in cyclopic fetuses versus those of normal fetuses. However, no histological or functional assessments of the pituitaries have been performed. Consequently, it is possible that the pituitary glands in the cyclopic fetuses were deficient in their ability to produce necessary hormones for normal development.

In summary, the results from this study confirmed that cyclopic fetuses are smaller than normal fetuses as well as less severely malformed

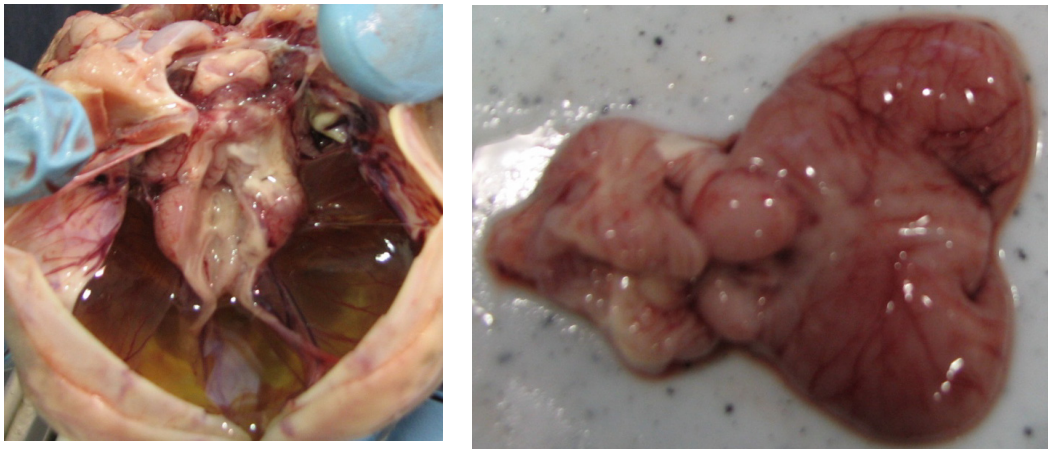

Figure 3. Fluid-filled brain cavity and brain from a cyclopic lamb.

fetuses. Additionally, we demonstrated that placental development in ewes with cyclopic fetuses is compromised. Due to the lack of brain and pituitary gland development in many of the cyclopic fetuses, it is quite likely that the lack of normal placental development in ewes with cyclopic fetuses is a result of insufficient contribution of the embryo during critical periods of placental development.

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