Elevated maternal cortisol leads to relative maternal hyperglycemia and increased stillbirth in ovine pregnancy.

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running head: Cortisol alters maternal glucose and pregnancy outcome in ewes
Abstract

In normal pregnancy cortisol increases, however further pathologic increases in cortisol are associated with maternal and fetal morbidities. These experiments were designed to test the hypothesis that increased maternal cortisol would increase maternal glucose concentrations, suppress fetal growth, and impair neonatal glucose homeostasis. Ewes were infused with cortisol (1 mg/kg/d) from day 115 of gestation to term; maternal glucose, insulin, oPL, estrone, progesterone, non-esterified free fatty acids (NEFA), β-hydroxybutyrate (BHB), and electrolytes were measured. Infusion of cortisol increased maternal glucose concentration, and slowed the glucose disappearance after injection of glucose; maternal infusion of cortisol also increased the incidence of fetal death at or near parturition. The design of the study was altered to terminate the study prior to delivery and post-hoc analysis of the data was performed in order to test the hypothesis that maternal metabolic factors predict the fetal outcome. In cortisol-infused ewes that had stillborn lambs, plasma insulin was increased relative to control ewes or cortisol-infused ewes with live lambs. Maternal cortisol infusion did not alter maternal food intake, or plasma NEFA, BHB, estrone, progesterone or placental lactogen concentrations, or alter fetal body weight, ponderal index, or fetal organ weights. Our study suggests that the adverse effect of elevated maternal cortisol on pregnancy outcome may be related to the effects of cortisol on maternal glucose homeostasis, and suggest that chronic maternal stress or adrenal hypersecretion of cortisol may create fetal pathophysiology paralleling some aspects of maternal gestational diabetes.

Key words: glucose, insulin, cortisol, pregnancy
Introduction

Maternal cortisol is increased in normal human pregnancy and is thought to contribute to the increase in maternal glucose concentration, as well as supporting increases in maternal plasma volume and cardiac output. In studies in our laboratory we have found that increasing maternal cortisol beyond the normal doubling in ovine pregnancy led to increased uterine blood flow and maternal and fetal glucose and lactate concentrations, whereas decreasing maternal cortisol to concentrations similar to those of nonpregnant ewes resulted in reduced uterine blood flow as gestation advanced (12; 13). Either increased or decreased maternal cortisol concentrations slowed the rate of fetal growth between 115 and 130 days gestation. In women with Cushing’s disease or syndrome in pregnancy, there is also an increased incidence of small for gestational age babies, as well as an increased risk of adverse pregnancy outcomes (26; 34). Maternal glucocorticoid treatment with dexamethasone or betamethasone is also known to reduce fetal growth and alter glucose in the offspring of sheep and rats (22; 23; 25). In order to test the hypothesis that more prolonged exposure to increased concentrations of the normally secreted corticosteroid, cortisol, in late gestation further perturbs maternal glucose homeostasis and further compromises fetal growth, we have extended our previous studies of elevated cortisol in late gestation to parturition. We hypothesized that a chronic increase in maternal cortisol would increase maternal plasma glucose and alter the maternal glucose response to glucose challenge. We expected that this would produce a lamb with altered glucose metabolism as a neonate. Surprisingly this model produced a very high incidence of fetal mortality and stillbirth. Maternal diabetes during pregnancy also increases the incidence of stillbirth (reviewed in (6)) and hence we have performed a post hoc analysis of these results to reveal possible relationships between maternal glucose homeostasis and pregnancy outcome.
Experimental Design

Pregnant black-faced ewes at 115 days of gestation were randomly assigned to the control group or the cortisol group (Solu-Cortef; hydrocortisone sodium succinate in sodium phosphate; Pfizer, Inc. NY, NY; 1mg/kg per day iv; cortisol group beginning on day 115 or 116 of pregnancy). Ewes were housed in pens in controlled light and humidity rooms beginning on or before day 108 of pregnancy. Ewes were fed a diet providing National Research Council (NRC) requirements for late gestation and had free access to water and salt blocks throughout the study. All use of animals was approved by the University of Florida Institutional Animal Care and Use Committee.

Surgery was performed under isoflurane anesthesia on ~115 days of gestation and maternal catheters were placed in both maternal femoral arteries and veins. A flow probe (6mm; 6PSS, Transonics, Ithaca, NY) was placed on the main uterine artery of the horn ipsilateral to the fetus and secured. The catheters and the flow probe cable exited on the left flank of the ewe and were placed in a pouch secured to the ewe with an elastic bandage retainer (Surgilast, Derma Sciences, Princeton, NJ). Ewes were treated with flunixin meglumine (2 mg/kg; Vedco Inc, St Joseph, MO) at the end of surgery and the day after surgery, and were treated with Polyflex (12.5-15 mg/kg; Boehringer Ingelheim, Hanford Pharmaceuticals, Syracuse, NY) for 5 days postoperatively. The initial experimental design was to study the pregnant ewes from 120 days gestation until term (expected at 144-146 d gestation), and to allow ewes to lamb and to study the lambs during early postnatal life. However, the initial groups of cortisol-infused ewes had a very high incidence of stillbirth (3 of 4 ewes, Figure 1 A and B). Therefore the study design was subsequently altered in order to perform a post-hoc analysis to test for changes in maternal physiology or fetal growth prior to death. Subsequent ewes in the study, 8 control (7 singleton and one twin) and 11 cortisol-infused (all singletons), were euthanized (Euthasol, Virbac Animal Health, Fort Worth, TX) at 143 days gestation or when signs of labor were apparent or stillbirth occurred. Subsequent analysis of the maternal data included the 13
control pregnancies (8 male and 6 female lambs including two sets of twins), and 15 cortisol treated pregnancies, 7 ending with live lamb/fetus at the time of euthanasia (all females) and 8 with either dead or stillborn fetuses (4 female and 4 male fetuses). Changes in pregnancy outcome were analyzed by Kaplan Meier survival analysis in the case of the first cohort of ewes allowed to deliver, and by rank sum test for the second cohort of ewes.

In all of the 28 ewes studied, a maternal blood sample was collected and uterine blood flow was measured for 30 min every 5 days from approximately 120 days to term or necropsy. All maternal blood samples (6mL) were collected in the morning under quiet conditions and with no restraint of the ewe. Uterine blood flow was measured using the Physiogear system (Transonics) which allows the ewes to move about the pen freely throughout the measurement period and was measured during a period following the blood collection in which no staff entered the animal room.

Plasma glucose and lactate were measured (YSI model 2300 Analyzer; Yellow Springs, OH) in samples collected in sodium fluoride and potassium oxalate. Plasma electrolytes (AVL 9180 Analyzer; Roche Diagnostics, Roswell, GA) including sodium, potassium, and calcium, packed cell volume (PCV), plasma protein (PP) (by refractrometry), plasma cortisol and progesterone (Siemens Coat-a-Count radioimmunoassay) were measured in samples collected in heparin, and plasma insulin (ovine insulin ELISA; ALPCO Diagnostics Inc, 80-INSOV-E01), ovine placental lactogen (oPL; RIA as described (14), nonesterified fatty acids (NEFA kit; Wako Diagnostics, Richmond VA) and β hydroxybutyrate (3-HB kit, Wako Diagnostics) were measured in samples collected in EDTA (BD Vacutainer, Becton Dickinson, East Rutherford, NJ). Changes in these variables with treatment and age were analyzed by 2 way analysis of variance (ANOVA) with correction for repeated measures over age (SPSS; IBM Corp, Armonk, NY) for the period of 120-135 days, as only a subset of ewes had samples at 140 days (13 control, 6 cortisol-infused with live lambs and 3 cortisol-infused with stillborn fetuses). Post hoc analysis of the data was also performed using 2 way ANOVA, but with segregation of the cortisol group by outcome at the time of delivery or necropsy. Mean uterine blood
flow data was also analyzed by 2 way ANOVA; data was excluded from 2 control ewes with twin fetuses as uterine blood flow is increased in twin pregnancies, and data was also excluded from 3 cortisol-infused ewes with incorrect placement of the uterine flow probe on the uterine artery contralateral to the fetus; therefore 11 control and 12 cortisol-infused ewes (6 with stillborn lambs and 6 with live lambs at necropsy/birth) were included in the analysis of uterine blood flow. The criterion for significance for all tests of baseline data was P<0.05 by two tailed test.

An intravenous glucose tolerance test (IVGTT) was performed at ~131-133 days of gestation. A bolus of glucose (0.4 g/kg based on body weight at 115 days) was injected into a venous catheter. Samples (2.5 ml each) for measurement of plasma glucose and insulin were collected at -5,-1, 2, 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, 120, 140, 160 and 180 minutes relative to the glucose injection. All glucose tolerance tests were performed in the morning before re-feeding of the ewes and the ewes were not allowed any access to feed during the 3 hours of study or for at least 1 hour prior to glucose injection. Because sheep are ruminants it is not possible to produce fasting conditions without withdrawal from food for approximately 72h; therefore this test was not done under fasting conditions. Blood samples were collected using catheters accessed from outside the pen so that ewes had free ability to move about the pen during the test period. The glucose and insulin responses over time were analyzed by two way ANOVA (group and time) corrected for repeated measures across time. The glucose data was also fit to a double exponential decay curve (Plasma glucose =y0+a*exp(-b*x)+c*exp(-d*x)) and differences among groups were analyzed by one way analysis of variance; in some ewes this fit was not significant and therefore the differences were compared only in those with significant fits (n=10 control, 6 cortisol-treated with live lambs and 5 cortisol-treated with stillborn lambs). The first phase insulin response, representing secretion of stored insulin, was analyzed as the area under the curve from 0 to 10 minutes; differences among groups were analyzed by one way analysis of variance. Post hoc analysis of the data was also
performed in which the cortisol group was segregated by outcome at the time of delivery or necropsy. The criterion for significance was $P<0.05$ by one tailed test.

Regression analysis was performed to test for relationships between mean plasma cortisol and insulin concentrations, and to test for associations of metabolites on insulin, and on insulin response to glucose injection. The effect of cortisol and metabolites on plasma insulin was modelled using both forward and backward regression analysis (Sigmaplot, Systat Software Inc, San Jose, CA) which yielded the same probability estimates for the variables tested. The effects of cortisol, metabolites, progesterone, estradiol and fetal body weight on uterine blood flow were also modelled using forward and backward stepwise regressions.
Results

Effects of cortisol on maternal cortisol

As expected, the infusion of cortisol increased maternal cortisol; this increase was not different in the ewes in the cortisol group who had live lambs at the time of necropsy and those whose lambs were stillborn or dead at necropsy (Table 1, Figure 2A and F); there were no significant changes over time in any group.

Effects on pregnancy outcome

There was an increased incidence of stillbirth apparent in the cortisol-infused ewes (8/9 ewes compared to 0/5 in the control group when those ewes were allowed to deliver (ie those not euthanized before the outcome of labor and delivery could be determined) were compared. In the first cohort of ewes, of the 5 control ewes (1 twin, 4 singleton pregnancies) all delivered live healthy lambs, whereas of the 4 cortisol infused ewes (all singleton pregnancies), 3 delivered stillborn lambs and the one lamb in this group which was live at birth was weak, unable to stand, and was euthanized (Figure 1 A and B; difference in outcome p<0.05). In the second cohort of 8 control ewes and 11 cortisol-infused ewes, euthanasia was performed once signs of labor occurred or by day 143; there were also differences between the groups in pregnancy outcome. In the control ewes, there was one live lamb that delivered and 7 live fetuses at the time of euthanasia; one fetus died during labor because of dystocia. In contrast, in the 11 cortisol-infused ewes, 5 had dead or stillborn fetuses, and 6 had live fetuses at euthanasia (Figure 1C and D). Pregnancy outcome (live vs dead) was also significantly altered by maternal cortisol infusion in this cohort. The cortisol-infusion advanced the time of delivery (140±1 for those 8 pregnancies with delivery, vs 145±1 for the 5 control pregnancies in which delivery occurred). The other pregnancies (6 cortisol-infused and 8 control ewes with live lambs at euthanasia) were terminated at 141±1 days gestation in the cortisol-infused group and at 143±1 days gestation in the control group.
There was no overall effect of maternal cortisol infusion on fetal body weight. The body weight of the stillborn lambs was also not significantly lower than that of the lambs that were live at birth or at euthanasia (Table 2) or as compared to control fetuses; this was true regardless of whether stillborn lambs delivered prior to 140 days were included, or whether data from control twins was excluded in the analysis. Placental weights were not available for those animals that delivered, so comparison is only possible between a subgroup of control and cortisol-infused fetuses in which necropsy was performed with intact placentomes. There were no differences in placental weight in those pregnancies (Table 2). There were also no significant differences in fetal organ weights between the live lambs of cortisol-infused and control ewes (Table 2) and no difference in ponderal index (weight/crown to rump length$^3$) among groups. Thus the chronic cortisol infusion did not result in either over-grown or stunted lambs.

Effects on maternal hormones, food intake, electrolytes and uterine blood flow

Maternal progesterone and estrone concentrations were not significantly changed by infusion of cortisol, nor were the changes over time significantly different (Figure 2, B and C). The plasma progesterone and estrone concentrations were also not predictive of pregnancy outcome (Figure 2, G and H).

Maternal food intake, calculated as the percent of the offered NRC requirement, was significantly greater overall in the cortisol-infused ewes as compared to control ewes (Figure 2D). This increase was significant overall only in the cortisol-infused ewes which had stillborn lambs, and appears to be related to increased food intake at 121 and 122 days of gestation (Figure 2I). Food intake in the cortisol-treated ewes with live lambs was actually significantly lower at several time points (126, 127, 128, 131, 132 d). As a measure of the maternal health during pregnancy, maternal body condition score and maternal plasma sodium, potassium and calcium concentrations were not different between the control and cortisol infused ewes (Table1). There was also no significant
overall effect of infusion of cortisol on packed cell volume or plasma protein (Table 1). Packed cell
volume and plasma potassium concentration were significantly reduced relative to 120d at 125d-135d
in control ewes, but this decrease did not occur in the cortisol-treated ewes. There were no
differences between cortisol-infused ewes with live fetuses/lambs as compared to stillborn
fetuses/lambs in electrolytes, PCV or plasma protein, suggesting there is no adverse effect on
maternal volume homeostasis related to pregnancy outcome.

Uterine blood flow was significantly increased overall with advancing gestational age; values
for flow were greater at 135 days and 140 days than at 120 days of gestation (Figure 2E). However
this increase was only significant in the control ewes. Uterine blood flow was not different overall
when all of the cortisol-treated ewes were compared with the control ewes (Figure 2E). However,
when the cortisol treated ewes were analyzed based on outcomes, the uterine blood flow in the
cortisol-treated ewes with live lambs was significantly increased relative to the uterine blood flow in
the control ewes or the cortisol-infused ewes who had either stillborn or dead fetuses at necropsy
(Figure 2J). There was no significant difference between flow in control ewes or cortisol-infused ewes
in which fetuses died in utero or at birth. Overall there was no significant relationship between
plasma cortisol concentrations and uterine blood flow, nor between fetal body weight and uterine
blood flow;; mean uterine flow was significantly related to maternal plasma estrone concentrations
(r=0.54) and maternal plasma NEFA concentrations (r=0.66, Table 3).

Effect of cortisol on maternal metabolites, insulin and placental lactogen

The infusion of cortisol increased maternal glucose; plasma glucose concentration was greater
in both the ewes with live fetuses or newborn lambs and those with dead fetuses or stillborn lambs
(Figure 3, A and D) as compared to control ewes. Plasma lactate concentration was not increased by
the infusion of cortisol, although the pattern of lactate over time was different among the groups; with
greater plasma lactate in both cortisol groups at 125 days gestation, but sustained higher lactate
levels only in those ewes whose fetuses died by the time of labor or euthanasia. (Figures 3C and F).

Plasma NEFA and β-hydroxybutyrate concentrations were not different among the groups (Table 1).

Overall, plasma insulin concentration was significantly increased by cortisol infusion but did not change significantly with advancing pregnancy (Figure 3B). Plasma insulin concentrations were increased in the cortisol-infused ewes that had stillborn or dead fetuses as compared to either the control ewes or to the cortisol-infused ewes that delivered live lambs or whose lambs were alive at the time of euthanasia. There was no difference in plasma insulin concentration between the control ewes and the cortisol-infused ewes who had live lambs at euthanasia (Figure 3E). There was no significant difference in insulin to glucose ratio among the groups, indicating that the increase in insulin is not disproportionate to the increase in glucose in the cortisol-infused ewes (Table 1).

Stepwise regression was used to assess the possible interaction of various metabolites and cortisol on maternal insulin concentrations. Both stepwise forward and stepwise backward regression of maternal average (120-140 days) insulin concentrations against plasma cortisol, glucose, lactate, NEFA and BHB concentrations indicated significant effects of cortisol on maternal plasma insulin concentrations (Table 3).

During the glucose tolerance test, overall glucose level in the cortisol group were significantly higher than those of the control group; plasma glucose was significantly increased at 60 and 80-180 minutes after injection of glucose (Figure 4A). Glucose was significantly increased at 20-120, 160 and 180 minutes in ewes treated with cortisol which had live fetuses or lambs as compared to control ewes. Glucose was significantly increased relative to controls at 160 and 180 minutes in those ewes with stillborn fetuses (Figure 4D). Overall there were differences between the two subsets of cortisol-infused ewes only at 2 min. Overall the glucose AUC was greater in cortisol-treated ewes regardless of pregnancy outcomes (Figure 5A and D).

The second phase glucose disappearance rate was significantly decreased in the ewes chronically infused with cortisol (Figure 5 C, F). This difference relative to control ewes was
significant in the cortisol-infused ewes with live lambs and those with stillborn lambs and did not differ between cortisol-infused ewes with different pregnancy outcome.

Plasma insulin concentrations in response to the injection of glucose were not different overall between the control and cortisol-treated ewes (Figure 4B); there was also no overall difference in the first phase insulin response (area under the curve from 0 to 10 minutes) among groups (Figure 5 B and E). However, the insulin response calculated over either 10 min or 30 minutes was exponentially (p<0.001) related to plasma cortisol concentrations at the start of the glucose tolerance test. One ewe in the control group had a cortisol concentration on the day of the test that was similar to those in the cortisol-infused group, when this animal was excluded from analysis, the insulin concentrations were significantly higher in ewes with stillborn lambs compared to control ewes at 10-20 and 40 min, and as compared to cortisol-infused ewes with live lambs at 5, 10 and 20-40 min (Figure 4E). The insulin to glucose ratio was also greater in the ewes with stillborn lambs than in control ewes at 10 minutes, and was greater than in cortisol-infused ewes with live lambs at 2, 5, and 20-40 minutes (Figure 4F).

Overall oPL values were not significantly different among the groups. There was a significant increase in oPL over gestation from 120-135 days; however the increase was significant only in the cortisol-treated ewes; these effects did not change when the ewes with twin fetuses (2 in the control group) were excluded from analysis. When the cortisol group was segregated by pregnancy outcome the increase with gestation was significant only in the cortisol-infused ewes with live lambs. The mean oPL concentration was significantly related to fetal weight (r=0.70, p<0.001, for fetuses at 139-144d gestation, n=25), but not to placental weight (r=0.38 for those for which euthanasia and necropsy occurred prior to delivery: n=11), or to mean plasma cortisol.
Discussion

The finding that chronic elevation of maternal cortisol dramatically increased the incidence of fetal death and/or stillbirth was unexpected based on our previous studies with shorter (10-14 days) duration of cortisol infusion ending at 130d gestation (13; 28). Although the cause of fetal demise is yet to be established in these fetuses, there appears to be an association with impaired maternal glucose tolerance and with maternal hyperinsulinemia. There also appears to be a close link between the timing of the demise and labor and/or delivery, with 6 of 8 fetal deaths appearing to occur after labor and delivery at term (ie between 140 and 146 days of gestation). Only two of the 7 live fetuses of the cortisol-infused ewes were collected from ewes in labor. Thus it is possible that the live fetuses in cortisol-infused ewes would have died if labor had been allowed to progress in those pregnancies. Therefore the separation of the two groups of cortisol-infused ewes may reflect only the timing of the onset of labor and a relative severity of maternal glucose intolerance, rather than ultimate fetal pathology.

In spite of the poor pregnancy outcome, there were no significant adverse effects other than hyperglycemia and hyperinsulinemia produced in the ewes by cortisol. We found no evidence of effects on maternal volume status, uterine blood flow, food intake, NEFA or placental hormones in the ewes with stillborn lambs as compared to control ewes, nor was fetal body weight significantly reduced by this increase in maternal cortisol. As in previous studies using other methods to assess uterine blood flow, we found that cortisol increased uterine flow in those ewes who went on to have live fetuses (13); in the present study this effect correlated with the tendency for this group to have higher plasma estrone concentrations, suggesting that the higher cortisol may stimulate greater placental estrogen production in this subgroup of ewes. However, since the plasma estrone concentrations were not significantly higher in either group of cortisol-infused ewes this observation requires study in future groups of animals. As postural effects in the ewe have been found to alter uterine flow (10), it is possible that we missed small changes in flow between the control ewes and
the ewes with stillborn lambs, although the incidence of ewes changing position during the recording period appears to be very low (6%) when the records of average flow over time were reviewed.

As expected, oPL concentrations correlated with fetal weight; maternal oPL is produced in placental binucleate cells, and is known to be positively correlated to placental weight and to fetal body weight (14; 16). Placental lactogen concentration is reduced with fetal death in utero (30); although the frequency of sampling in this study was not sufficient to observe a decrease in oPL at fetal death, there was no significant association with cortisol, suggesting that there was no major placental pathology in these pregnancies. Infusions of cortisol to the fetus have been found to decrease binucleate cell number stained by oPL immunohistochemistry (36), however the concentrations of cortisol achieved in that study were meant to simulate the late gestation surge in fetal cortisol and therefore are higher than the concentrations achieved in this study in which maternal and fetal cortisol concentrations were approximately doubled at 130d (7; 13).

In cortisol infused ewes the rate of glucose disappearance after administration of a bolus of glucose was slowed. In the present study the altered basal glucose and glucose disappearance rate suggests relative glucose intolerance in the cortisol- infused ewes as compared to normal pregnant control ewes. This effect of cortisol is not surprising, as patients with Cushing’s disease have increased glucose response to oral glucose challenge and reduced glucose disposal during euglycemic clamps with insulin infusions (24). Treatment with a glucocorticoid receptor blocker has been shown to reduce the insulin resistance associated with Cushing’s syndrome and adrenal incidentalomas (4; 8). A recent report also suggests that some women with gestational diabetes may have elevated plasma cortisol (18).

In contrast to the effect on plasma glucose, which was evident in both the cortisol-infused ewes with live and with stillborn lambs at necropsy, there was a significant difference in basal plasma insulin only in the cortisol-infused group whose fetuses died in utero or at birth. Overall the plasma insulin concentrations correlated positively to cortisol. Although glucocorticoids are generally thought
to reduce glucose stimulated insulin secretion (1; 5; 33), a chronic decrease in glucocorticoids also inhibits insulin secretion (15). The increase in cortisol in the present study may not be sufficient to inhibit insulin production, and the association with cortisol in the present study may be indirect, reflecting pancreatic compensation for chronic glucose intolerance. The basal insulin to glucose ratio was not significantly higher in the cortisol-infused ewes, including those with stillborn lambs, suggesting that glucose-stimulated insulin secretion is not disrupted by this concentration of cortisol, and that increases in insulin are concordant with the degree of hyperglycemia. The first phase insulin response was not altered in the cortisol- infused ewes, also suggesting that glucose-stimulated secretion of stored insulin was not impaired. Late gestation ewes show a reduced insulin response to glucose challenge compared to nonpregnant ewes (27), but this insulin response was not altered in the cortisol-infused ewes as compared with normal control pregnant ewes. There was no relationship between either plasma NEFA or plasma oPL and the insulin response, although both of these have been suggested as mediators of altered insulin secretion in pregnancy (1; 27). The higher plasma insulin at later time points in response to glucose challenge, which was significant only at some time points when the cortisol group was segregated based on adverse pregnancy outcome, may reflect relatively greater insulin resistance in the sub-group of cortisol-infused ewes who later have stillborn lambs.

In summary, these studies suggest an association between death at or near term and maternal metabolic dysfunction secondary to maternal hypercortisolemia. However no association with placental or fetal weight or uterine blood flow was found.

Perspectives

In humans stillbirth is frequently associated with maternal hypertension or diabetes (3; 6; 21; 32) but is also increased in women with Cushing’s disease (17; 26; 34; 35) and maternal stress or depression (8; 10; 16; 37) in which maternal cortisol would be chronically increased. Our study
suggests that the adverse outcomes in ovine pregnancy with hypercortisolemia may be related to the metabolic effects of cortisol in the mother and/or fetus. Although pregnancy loss in humans with elevated cortisol or diabetes may be multifactorial, changes in cardiac size and/or function in the fetus has been suggested as one factor contributing to late term fetal loss in these pregnancies (34; 35; 41). While it is enticing to hypothesize that the dramatic increase in stillbirths found in the present study is related to the changes in the fetal heart previously observed at 130 days of gestation in this model (8; 31) and that have been found with glucocorticoid administration (12; 20; 32; 37; 43), there is no clear association with heart weight in this group of fetuses. However, in gestational diabetes there may be fetal diastolic dysfunction independent of hypertrophy (2). In diabetic pregnancies, fetal lactic acidosis and hypoxia have both been reported near term, and bradycardic episodes, ST segment depression and fetal acidemia have been observed during labor in fetuses of both gestational and pregestational diabetic mothers (31; 37; 38) as well as heart failure with abnormal ECG during labor (19). An increased incidence of fetal bradycardiac responses to fetal stress occur in women demonstrating high anxiety during pregnancy (20). It is not known if acidemia and bradycardia occur during labor in fetuses with Cushing’s disease, however in ovine fetuses exposure to glucocorticoids appears to increase the bradycardia and acidemia during a hypoxic episode (9; 11). Despite the apparently normal heart size in the cortisol-treated fetuses at term, we have, in fact, found significant differences in gene expression in the septa collected from the hearts of the cortisol-infused fetuses who were live at the time of euthanasia as compared to control fetuses. Pathway analysis of the differences in gene expression indicate differences in nutrient pathways and in genes associated with mitochondria, suggesting altered metabolism in the septum secondary to excess cortisol exposure (29). Among the genes that were significantly up-regulated was pyruvate dehydrogenase kinase 4 (PDK4), the inhibitor of pyruvate dehydrogenase, needed for pyruvate conversion into acetyl Co-A and Kreb’s cycle activity. Mitochondrial DNA was reduced, suggesting mitochondrial loss.
In keeping with the possibility that maternal hypercortisolemia, with resultant maternal hyperglycemia may alter fetal metabolism, the fetal loss is not associated with placental, fetal or cardiac size, but appears to be temporally related to labor and delivery. If we used normal uterine flow as an indicator of onset of labor and of fetal viability (since flow depends in part on estradiol levels which reflect placental estradiol production), and taking into consideration the condition of the fetal tissues at euthanasia, it appeared that the fetal deaths occurred on the day of the euthanasia, or within hours of delivery, and therefore near or at term. Thus the high incidence of fetal death in the intrapartum period suggests a functional pathophysiology in the fetuses revealed by the stress of labor and/or delivery which maybe an analogous metabolic dysfunction to that observed in some human pregnancies complicated by maternal hypercortisolemia and/or maternal hyperglycemia.
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Figure Legends

Figure 1: Pregnancy outcomes in control ewes or ewes infused with cortisol (1 mg/kg/d) starting at 115d gestation. Panels A and B are from studies in which ewes were allowed to deliver: at each gestational age, gray bars represents the number of ewes with in utero fetuses, white bars the number of ewes who delivered live lambs at that gestational age, and black bars the number of ewes delivering a dead lamb at that age. Panels C and D are the results in studies in which the ewe was necropsied at the time of labor or planned necropsy or early delivery occurred. Bars are as in Panels A and B, gray bars indicate ewes with in utero fetuses, white bars indicate ewes with live birth at that gestational age, striped bars indicate ewes with live lambs at euthanasia at that gestational age, and black bars represent the number of ewes with a stillbirth determined at that age, either as a result of delivery of a dead lamb or of discovery of dead lamb at necropsy Note that bar height decreases over gestational age as ewes delivered or the necropsies were performed.

Figure 2: Plasma cortisol (A, F), progesterone (B, G), estrone (C, H), food intake (D, I) and uterine blood flow (E and J) in control ewes (open circles, n=13) or ewes infused with cortisol (1 mg/kg/d from 115 days to term; closed circles; panels A-E, n=15). Values in control and cortisol treated ewes segregated by pregnancy outcome are shown in Panels F-J: cortisol-treated ewes delivering live lambs or with live lambs at the time of euthanasia (closed gray squares, n=7) or those delivering stillborn lambs or with dead lambs at necropsy (closed black triangles, n=8). Data are shown as mean values ± SEM. * indicates overall differences between all cortisol-treated compared to control ewes (A-E) or ewes treated with cortisol with live lambs vs control ewes (F-J). ^ indicates significant overall difference between ewes with stillborn lambs vs control ewes (F-J). Uterine flow data excludes 2 ewes in the control group with twins and two ewes in the cortisol-treated group with improper placement of the flow probe.
Figure 3: Plasma glucose (A, D), insulin (B, E) and lactate (C, F) concentrations in control and cortisol-infused ewes. Symbols and n's are as indicated in the legend to Figure 2. # indicates differences between cortisol-treated ewes with live vs stillborn lambs.

Figure 4: Plasma glucose (A, C) and insulin (B, D) concentrations in response to intravenous injection of 0.40 g glucose/kg in control and cortisol-treated ewes. Values depict mean ±SEM for plasma glucose and insulin concentrations. Panels A and B depict responses in control and all cortisol treated ewes; panels C and D depict responses in control ewes and cortisol-treated ewes who had either live or stillborn lambs at the time of euthanasia. * and ^ indicate differences compared to control ewes, as in Figure 2. # indicates significant difference between cortisol-treated ewes with stillborn lambs as compared to those with live lambs. Results include 12 control and 15 pregnant ewes, 7 with live fetuses at the time of euthanasia or birth, and 8 with dead fetuses or stillborn lambs.

Figure 5: Total area under the curve (AUC) data for glucose to 180 min (panels A and D), for insulin to 10 min (panels B and E) and estimates of the slow glucose disappearance rate (parameter d of 5 parameter fit: $y=y_0+a\exp(-b\times x)+c\exp(-d\times x)$) (panels C and F). Upper panels (A-C) depict values from control ewes (white bars, n=12) vs all cortisol-infused ewes (black bars, n=15); lower panels (D-F) are data with results in cortisol-infused ewes segregated by pregnancy outcome (gray bars, live fetuses at euthanasia or delivery, n=7; black bars, stillborn, n=8). Symbols to indicate significance are used as indicated in Figure 2.
Table 1; Mean values from 120-140 days gestation in control and cortisol-infused pregnant ewes

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<th>Control (n=13)</th>
<th>All Cortisol-Treated (n=15)</th>
<th>Cortisol-Treated with live lambs (n=7)</th>
<th>Cortisol-Treated with stillborn or dead lambs (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body condition score</td>
<td>3.1±0.1</td>
<td>3.1±0.1</td>
<td>3.1±0.1</td>
<td>3.1±0.1</td>
</tr>
<tr>
<td>Cortisol (ng/ml)</td>
<td>9.1±1.3</td>
<td>17.4±1.2*</td>
<td>18.5±1.8*</td>
<td>16.4±1.7*</td>
</tr>
<tr>
<td>oPL (ng/ml)</td>
<td>326.9±44.0</td>
<td>275.2±29.0</td>
<td>310.8±42.2</td>
<td>244.0±39.0</td>
</tr>
<tr>
<td>Plasma NEFA (µM)</td>
<td>219±27</td>
<td>186±22</td>
<td>216±40</td>
<td>160±19</td>
</tr>
<tr>
<td>Plasma BHB</td>
<td>67.8±10.6</td>
<td>81.6±11.7</td>
<td>73.3±15.0</td>
<td>88.2±13.0</td>
</tr>
<tr>
<td>Plasma lactate (mg/dl)</td>
<td>6.0±0.8</td>
<td>8.0±0.8</td>
<td>7.0±0.7</td>
<td>8.9±1.3</td>
</tr>
<tr>
<td>Plasma Insulin/Glucose</td>
<td>0.021±0.003</td>
<td>0.027±0.003</td>
<td>0.025±0.004</td>
<td>0.029±0.004</td>
</tr>
<tr>
<td>PCV (%)</td>
<td>29.7±0.9</td>
<td>29.5±0.6</td>
<td>29.4±1.1</td>
<td>29.3±1.1</td>
</tr>
<tr>
<td>Plasma Protein (mg/dl)</td>
<td>6.8±0.1</td>
<td>7.0±0.1</td>
<td>7.0±0.1</td>
<td>7.0±0.2</td>
</tr>
<tr>
<td>Plasma Na⁺ (mEq/L)</td>
<td>146.8±0.3</td>
<td>146.0±0.5</td>
<td>145.7±0.6</td>
<td>146.4±0.6</td>
</tr>
<tr>
<td>Plasma K⁺ (mEq/L)</td>
<td>4.18±0.05</td>
<td>4.23±0.03</td>
<td>4.27±0.04</td>
<td>4.19±0.04</td>
</tr>
<tr>
<td>Plasma Ca²⁺ (mEq/L)</td>
<td>4.93±0.11</td>
<td>4.93±0.06</td>
<td>4.95±0.07</td>
<td>4.91±0.09</td>
</tr>
</tbody>
</table>

Values are mean ±SEM calculated from the mean value in each ewe from samples collected between 120 and 140 days (4-5 per ewe). 3HB data are from 12 control, 6 ewes with live lambs/fetuses and 8 ewes with stillborn or dead fetuses. *Indicates differences from control.
### Table 2: Fetal and Placental Growth Indices

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>All Cortisol-Treated</th>
<th>Cortisol-Treated with live lambs</th>
<th>Cortisol-Treated with stillborn or dead lambs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placental weight (n)†</td>
<td>461.7±95.3 (5)</td>
<td>422.9±41.3 (6)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Fetal weight (n) ††</td>
<td>5222±365 (15)</td>
<td>4723±335 (15)</td>
<td>5450±274 (7)</td>
<td>4428±570 (6)</td>
</tr>
<tr>
<td>(without twins; n)</td>
<td>(5280±433; 11)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ponderal Index (n)</td>
<td>29.3±1.9 (14)</td>
<td>24.8±1.8 (11)</td>
<td>26.3±2.6 (7)</td>
<td>22.0±1.2 (4)</td>
</tr>
<tr>
<td>(without twins; n)</td>
<td>(29.2±2.7; 10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain/body weight (x 10²)</td>
<td>1.12±0.11</td>
<td>1.01±0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart/body weight</td>
<td>0.641±0.027</td>
<td>0.676±0.036</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung/body weight (x 10²)</td>
<td>3.10±0.20</td>
<td>3.13±0.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver/body weight (x 10²)</td>
<td>2.42±0.16</td>
<td>2.33±0.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney/body weight (x 10³)</td>
<td>0.231±0.011</td>
<td>0.271±0.016</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adrenal/body weight (x 10⁵)</td>
<td>5.44±0.64</td>
<td>5.93±0.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Perirenal adipose/body weight (x 10²)</td>
<td>0.730±0.062</td>
<td>0.764±0.030</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Organ weights are mean ±SEM for data from live fetuses or newborns; n=8 control and n=7 for cortisol-infused groups. †data for placenta from ewes that gave birth (live or dead) not available; ††data on fetal weights does not include weights of 2 stillborn fetuses born at 132 and 137 d (mean ±SEM, weight including these lambs is 4086±490 for stillborn lambs). There were no significant differences between or among groups in any of these variables.
Table 3: Modeling of maternal plasma insulin and uterine flow relationships to maternal cortisol and metabolic factors; probability calculations (p) and regression coefficient (r) calculated using stepwise forward or reverse regression analyses (*indicates <p.05, nd=variables not included in the regression analysis)

<table>
<thead>
<tr>
<th></th>
<th>Insulin p</th>
<th>Insulin r</th>
<th>UBF p</th>
<th>UBF r</th>
</tr>
</thead>
<tbody>
<tr>
<td>cortisol</td>
<td>&lt;0.001*</td>
<td>0.587</td>
<td>0.642</td>
<td></td>
</tr>
<tr>
<td>glucose</td>
<td>0.095</td>
<td>0.151</td>
<td></td>
<td></td>
</tr>
<tr>
<td>lactate</td>
<td>0.285</td>
<td>0.892</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NEFA</td>
<td>0.009*</td>
<td>0.719</td>
<td>0.005*</td>
<td>0.660</td>
</tr>
<tr>
<td>BHB</td>
<td>0.769</td>
<td>0.545</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCS</td>
<td>0.841</td>
<td>0.231</td>
<td></td>
<td></td>
</tr>
<tr>
<td>insulin</td>
<td>-</td>
<td>0.749</td>
<td></td>
<td></td>
</tr>
<tr>
<td>progesterone</td>
<td>nd</td>
<td>0.940</td>
<td></td>
<td></td>
</tr>
<tr>
<td>estrone</td>
<td>nd</td>
<td>0.002*</td>
<td>0.544</td>
<td></td>
</tr>
<tr>
<td>oPL</td>
<td>nd</td>
<td>0.362</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetal weight</td>
<td>nd</td>
<td>0.562</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


