Glucose is essential for fetal growth and is transported through the placenta from maternal blood to the fetus during pregnancy. Facilitated fetal cortisol secretion causes an increase in the concentration of glucose in maternal blood by its insulin antagonistic action [10]. Furthermore, glucocorticoid is involved in regulating the expression of placental glucose transporter (GLUT) 1 and 3 mRNA levels, the action was not observed in clone pregnancy. These results raise the possibility of facilitation of glucose transportation through the placenta to meet increased nutritional requirements of overgrown clone fetuses.

Key words: Clone, Cortisol, Glucose, GLUT, Placenta

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ters to investigate whether clone fetuses secrete cortisol normally during late pregnancy.

The concentration of glucose in the maternal circulation is affected by nutritional repletion of pregnant animals [12], and a reduced concentration correlates with insufficient fetoplacental development, leading to neonatal weakness [13]. The nutritional requirements of fetuses also affect the maternal glucose level, and twin-bearing cows show reduced glucose concentrations that might be induced by the increase of the placentome number and/or consumption of nutrition by the fetuses [14, 15]. Therefore, it is expected that clone pregnancies with fetal overgrowth and neonatal death involve changes in the maternal glucose level that accompany fetal abnormality.

Here, correlations between the glucose concentration in maternal blood and abnormality of clone calves were examined through analyses of the glucose concentration in maternal blood, the birth weights of clone calves and GLUT mRNA expression levels in the term placenta. In addition, we examined the influence of dexamethasone, a synthetic glucocorticoid used to induce parturition as an alternative to fetal cortisol, on GLUT mRNA expression in the term placenta.

Materials and Methods

Animals and treatment

Clones were produced by nuclear transfer using fibroblasts from the skin of male Japanese black calves for donor nuclei. The procedures for production of clones were described previously [16]. Control calves (Japanese black, Angus, Hereford and crossbreed) were produced by transferring embryos fertilized in vivo into recipient cows. The calves were born at the Hokkaido Animal Research Center (HARC) and the National Livestock Breeding Center Tokachi Station (NLBC). The production of cloned cattle was approved by the Animal Experiment Committee. In both facilities, the recipient cows were fed to investigate whether clone fetuses secrete cortisol normally during late pregnancy.

The average length of gestation in the cows and calves used for blood collection was 284 days (n=22) for the Clone group and 283 days (n=6) for the DEX group. The average periods of gestation in the cows used to collect samples of placental tissue were 286 days (n=14), 287 days (n=5), 286 days (n=4) and 293 days (n=4) for the Clone, DEX, PG and SP groups, respectively.

Glucose concentrations

Plasma glucose concentrations were measured with Synchron system Glucose Reagent (reference number 442640, Beckman Coulter, Tokyo, Japan) and a Synchron CX5 Delta Clinical System (Beckman Coulter).

Reverse transcription and real-time PCR

Total RNA was individually isolated from COT and CAR using ISOGEN (Nippon Gene, Toyama, Japan) and reverse transcribed using a QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions.

Real-time PCR was performed (run in duplicate) using a QuantiTect SYBR Green PCR Kit (Qiagen) and a Chromo 4 Real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA) with the following primers: 5’-AGCGTCATCCTCTCATCCCGACG-3’, forward, and 5’-CCACAATGCTCAGGTTAGGAC-3’, reverse, for GLUT1 and (annealing temperature: 58 C [17]); 5’-ATG-GCAGTTGGCTGTTGTC-3’ and 5’-TCTCTGAGCTGAA-GAGAATGC-3’, for GLUT3 (58 C [17]); 5’-CATCTTGTG-TCACGGTGTG-3’ and 5’-CTGAACGACAGAATTGGT-3’ for Glucocorticoid receptor α (GC-Rα; 55 C [18]); and 5’-GAA-GACTTTGCCATGGCC-3’ and 5’-CTGAGGCTGTCATCCACC-3’ for GAPDH (60 C [19]). The thermal cycling conditions included activation of HotStarTaq DNA Polymerase at 95 C for 15 min, followed by 45 cycles of denaturation at 94 C for 15 sec; annealing of primers at different temperatures for 30 sec; and elongation at 72 C for 30 sec. To quantify the mRNA abundance, standard curves for each gene were generated by serial dilution of a known quantity of purified RT-PCR products. The relative difference in the initial amount of each mRNA species was determined by comparing the Ct values. We confirmed the melting curve for detecting the SYBR Green-based objective amplicon because SYBR Green also detects double-stranded DNA, including primer dimers, contaminating DNA and PCR products from mis annealed primers. The expression of each gene relative to that of GAPDH mRNA was calculated for adjustment of the amount of RNA used in the RT-PCR.
Statistical analysis
All results are presented as means ± S.E.M. Statistical analyses were performed using the Student’s t-test and one-way ANOVA followed by Scheffé’s test. The relationships between maternal glucose concentrations and birth weights of calves were evaluated using Pearson’s correlation coefficient.

Results

Glucose concentration
As shown in Table 1, the maternal plasma glucose concentrations were unchanged between days 257 and 271 of gestation, but increased significantly at term in both the Clone and Control groups. The plasma glucose concentrations were significantly lower in the Clone group than in the Control group during the period examined. The increase in the plasma glucose concentrations at term was lower in the Clone group than in the Control group and there was a significant difference in the ratio of Term to day 257 of gestation. There was no difference in the plasma glucose concentrations of calves at birth between the Clone and Control groups.

GLUT1, GLUT3 and GC-Rα mRNA expression in the placentome
The GLUT1, GLUT3 and GC-Rα mRNA levels in the COT did not differ among the Clone and Control (DEX, PG and SP) groups (Fig. 1A, C and E).

GLUT1 mRNA expression in the CAR was significantly higher.
in the Clone and PG groups than in the SP group (Fig. 1B). GLUT1 mRNA expression in the CAR tended to be lower in the DEX group than in the Clone and PG groups. GLUT3 mRNA expression in the CAR was significantly higher in the Clone group than in the DEX and SP groups (Fig. 1D). GLUT3 mRNA expression in the CAR was also significantly higher in the PG group than in the SP group. Although GC-Rα mRNA expression in the CAR was significantly higher in the DEX group than in the SP group, there was no difference among the Clone, DEX and PG groups (Fig. 1F).

Correlation between maternal glucose concentrations and birth weights

The birth weight of the Clone group (48 ± 3 kg) was significantly higher (P<0.01) than that of the DEX group (27 ± 1 kg).

In the Clone group, the maternal plasma glucose concentrations at day 257 of gestation (r = –0.584, P<0.01) and term (r = –0.549, P<0.01) were correlated significantly with birth weight, and those at day 271 of gestation showed poor correlation (r = –0.286, P= 0.198; Fig. 2).

In contrast, the maternal plasma glucose concentrations of the Control group (DEX) at days 257 (r = –0.084, P=0.875) and 271 (r = 0.175, P=0.740) of gestation and term (r = –0.479, P=0.336) did not show significant correlation with birth weights.

Discussion

In this study, low maternal plasma glucose concentrations in clone pregnancies were associated with the incidence of LOS. This relationship was observed from 4 weeks before to just before parturition. A previous report revealed that overgrown clones were observed by day 100 of gestation when the facilitation of fetal cortisol secretion had not begun [20]. Additionally, clone calves showed normal plasma glucose concentrations at birth. Therefore, the decrease of the glucose concentration in the maternal circulation during late pregnancy seems to be due to excess glucose requirements of overgrown fetuses as in a twin pregnancy with normal fetuses rather than insufficiency of the insulin antagonistic action by fetal cortisol. Although it should be noted that the concentration of glucose in maternal blood is affected by the feeding conditions of herds, the current data raise the possibility that maternal glucose levels allow a rough estimate of the occurrence of LOS at least after the 4th week pre-parturition. Because dystocia caused by a lack of readiness for birth in pregnant cows and LOS may result in mortality of perinatal clones, information on birth weight would be useful to decide on a cesarean delivery.

The difference in GLUT mRNA abundance among the controls, which were induced to undergo parturition with/without dexamethasone or delivered spontaneously, indicated consecutive downregulation of placental GLUT mRNA expression as parturition proceeded. Thus, the mRNA levels of GLUT1 and GLUT3 in caruncles decreased on administration of dexamethasone compared with the induction of parturition using only prostaglandin Fα and estriol, and the lowest levels were observed after spontaneous delivery. Therefore, it was supposed that dexamethasone caused the decrease in placental GLUT mRNA expression and that endogenous cortisol suppressed it more strongly. This assumption is consistent with a previous study showing that glucocorticoid down-regulates GLUT1 and GLUT3 mRNA and protein expression in human trophoblast cells and the rat placenta [11].

Placental GLUT mRNA expression in clone pregnancy did not decrease at parturition, despite the administration of dexamethasone. The ongoing expression implied an increase of glucose transportation to the clone fetuses. Clone and control pregnancies in which parturition was induced with or without dexamethasone showed similar placental GC-Rα mRNA abundance, except for a decrease in GC-Rα mRNA abundance in the controls that delivered spontaneously. Therefore, the differences in placental GLUT mRNA levels seemed to be caused by the glucocorticoid levels in the placentome rather than disruption of signal transduction through the receptor.

The distribution of GLUT1 and GLUT3 in placentomes was studied in detail by Wooding et al. [12] using immunohistochemistry. GLUT1 was detected in the basolateral plasmalemmas of both the trophoblast lining the fetal villi and the caruncle epithelium.
GLUT3 was detected in interdigitated areas of the trophoblast and the caruncle epithelium. We extracted mRNA from cotyledons and caruncles, respectively, and quantified GLUT mRNA abundances. Cotyledons showed less mRNA than caruncles, and the levels in cotyledons were constant among animals, mainly because the samples contained a fetal membrane overlying the placenta. In contrast, the abundance of GLUT mRNA was detected in caruncle samples including the placental labyrinth, which was occupied by the fetal villi and the caruncle epithelium.

The concentration of glucose in maternal blood remains stable during late pregnancy and then rises sharply before parturition [13]. This prepartum rise is caused by the insulin antagonistic activity of glucocorticoid [10]. Clone pregnancy also showed an increase in the plasma glucose concentration just before parturition; however, the increase was modest. It is possible that an insufficiency of fetal cortisol secretion at parturition caused the outcome, but the concentration of cortisol and the function of the hypothalamic-pituitary-adrenal axis in clone fetuses need to be confirmed in future studies.

In summary, the maternal plasma glucose concentration correlated inversely with birth weights of clone calves, and suppression of placental GLUT mRNA levels by administration of dexamethasone was not observed in clone pregnancy. These results raise the possibility of facilitation of glucose transportation through the placenta to meet the nutritional requirements of overgrown clone fetuses.

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