—Original Article—

Prepartum Maternal Plasma Glucose Concentrations and Placental Glucose Transporter mRNA Expression in Cows Carrying Somatic Cell Clone Fetuses

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Abstract. In this study, the plasma glucose concentrations of cows carrying a somatic cell clone fetus during late pregnancy and placental glucose transporter (*GLUT*) mRNA levels at parturition were examined. Parturition was induced using dexamethasone, prostaglandin $F_{2\alpha}$ and estriol in cows bearing a clone (Clone) or a fetus fertilized *in vivo* as a control (DEX). Plasma glucose concentrations were measured in the cows (days 257 and 271 of pregnancy and at parturition) and newborn calves. Cotyledon and caruncle tissues removed just after parturition were used for mRNA extraction. Expression of mRNA was also analyzed in control cows that were induced to undergo parturition without dexamethasone (PG) or that spontaneously delivered (SP). The glucose concentrations of the Clone group were significantly low at all points examined, but those of the calves were normal. The increase in the maternal glucose concentration from day 257 to parturition was significantly lower in the Clone group. Glucose concentrations were negatively correlated with birth weight for clones (day 257; $r=-0.584$, day 271; $r=-0.286$, parturition; $r=-0.549$). There was no difference in mRNA levels in the cotyledons among the animals examined. In the caruncles, the Clone and PG groups showed significantly higher *GLUT1* and *GLUT3* mRNA levels than the SP group, and the *GLUT3* mRNA level was significantly higher in the Clone group than in the DEX group. The glucocorticoid receptor α mRNA level was significantly lower in the SP group than in the DEX group. Although spontaneous parturition and administration of dexamethasone suppressed the placental *GLUT* 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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Cattle cloning by somatic cell nuclear transfer has been expected to improve the efficiency of animal breeding and to have advantages in the efficient production of genetically-engineered animals. However, low production efficiency remains a barrier to the practical application of the technology. A high incidence of gestational loss in early to mid-pregnancy is the most remarkable feature of the cloning of cattle [1], but problems in the peripartum period, such as delayed parturition, large offspring syndrome (LOS) and neonatal death, are also factors in decreasing the production efficiency [1–4]. LOS and poor sign of parturition in pregnant cows cause dystocia leading to losses of dams and newborn calves.

Cortisol, a glucocorticoid secreted from the fetal adrenal cortex, plays an important role in the initiation and progression of parturition [5, 6]. The secretion of cortisol from the fetal adrenal cortex is facilitated particularly during the last week of gestation and reaches a peak just before parturition [5]. In the placenta, cortisol promotes the synthesis of estrogens that induce relaxation and dilation of the

birth canal [7]. Furthermore, cortisol downregulates fetal growth and stimulates the maturation of those organs essential for extra-uterine survival, such as the lungs and thyroid [8]. Previously, we reported an insufficient increase of the prepartum estrogen concentration in pregnant cows carrying a clone fetus [9]. LOS and respiratory distress syndrome are also frequently observed in neonatal clone calves [2]. These findings imply that disruption of cortisol secretion in clone fetuses is a principal cause of the problems in the peripartum period. Chavatte-Palmer *et al.* [3] reported that the responsiveness of the adrenocortical tissue to adrenocorticotrophin in clone calves is normal, and we have also shown that clone calves had the ability to secrete cortisol at parturition [9]. However, the secretion of cortisol in prepartum clone fetuses is not fully understood.

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 [11], influencing the amount of glucose transported to the fetus and fetal growth. Therefore, the glucose concentration in maternal blood and placental *GLUT* expression could be useful as param-

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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 placental 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) 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 grass hay and grass silage, and the herds were comprised considering body constitution and social hierarchy.

The induction of parturition in cows carrying a clone (Clone) was achieved with 20 mg i.m. dexamethasone (Denka Pharmaceutical, Kawasaki, Japan), followed by prostaglandin $F_{2\alpha}$ (PGF_{2 α} ; 0.75–1 mg i.m. cloprostenol [Resipron-C, Aska Pharmaceutical, Tokyo, Japan] or 25 mg i.m. tromethamine dinoprost [Pronalgon-F injection, Pharmacia, Tokyo, Japan]) and 20 mg i.m. estriol (Holin, Teikoku Zoki, Tokyo, Japan) 24 h later. Control cows were treated in the following three ways: administration of dexamethasone followed by PGF_{2 α} and estriol 24 h later (DEX), PGF_{2 α} and estriol without dexamethasone (PG) and spontaneous parturition without induction of parturition (SP). Calves were delivered 1 or 2 days after the administration of PGF_{2 α} and estriol. Some clones were delivered by cesarean section after the induction of parturition. Beef breeds were used as recipient cows for collection of blood (HARC, Angus and crossbreed; NLBC, Japanese black, Japanese brown and crossbreed) and placentomes (HARC; Japanese black, Angus, Hereford and crossbreed).

Sample collection

Venous blood samples of the Clone group were taken at days

257 and 271 of gestation and just before parturition. Venous blood samples of the DEX group were taken on the same days of gestation as for the control. Venous blood samples of the calves were taken just after parturition. Plasma was harvested by centrifugation and stored at –20 C until the analyses.

Placentomes were collected manually, immediately after vaginal delivery and cesarean section, through either the birth canal or the incision made in the uterus. They were separated manually into two portions, the cotyledon (COT) and the caruncle (CAR), and stored at –80 C prior to the extraction of RNA.

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'-AGCGTCATCTTCATCCCAGC-3', forward, and 5'-CCACAATGCTCAGGTAGGAC-3', reverse, for *GLUT1* and (annealing temperature: 58 C [17]); 5'-ATGCGAGTGGCTGGTTGTTTC-3' and 5'-TCCTGAGCTGAA-GAGAATGTCC-3', for *GLUT3* (58 C [17]); 5'-CCATTCTGT-TCACGGTGTG-3' and 5'-CTGAACCGACAGGAATTGGT-3' for *Glucocorticoid receptor α* (*GC-R α* ; 55 C [18]); and 5'-CCA-GAAGACTGTGGATGGCC-3' and 5'-CTGACGCCTGCTTCA-CCACC-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 misannealed 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 $\pm$ 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-R α mRNA expression in the placentome

The GLUT1, GLUT3 and GC-R α 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

Table 1. Maternal plasma glucose concentrations in late pregnancy and plasma glucose concentrations of newborn calves

	Animals	
	Clone	Control
Number of dams/calves	22	6
Maternal glucose		
Day 257 (mg/dl)	56.2 $\pm$ 1.1	62.2 $\pm$ 1.0 **
Day 271 (mg/dl)	52.9 $\pm$ 1.8	62.2 $\pm$ 1.6 **
Term (mg/dl)	81.8 $\pm$ 3.8 †	106.2 $\pm$ 11.8 **,†
Term / Day 257	1.455 $\pm$ 0.058	1.705 $\pm$ 0.185 *
Term / Day 271	1.562 $\pm$ 0.068	1.712 $\pm$ 0.198
Calf glucose (mg/dl)	45.4 $\pm$ 3.5	47.5 $\pm$ 4.2

*P < 0.05, ** *P* < 0.01: significant differences between the Clone and Control groups. † *P* < 0.01: significant difference compared with the maternal plasma glucose concentrations at days 257 in each animal.

Fig. 1. GLUT1, GLUT3 and GC-R α mRNA levels in term placental tissues. The abundance of mRNA in cotyledonary (A, C and E) and caruncle (B, D and F) tissues was analyzed by real-time RT-PCR. Cows carrying a somatic cell clone (Clone) were induced to undergo parturition with an administration of dexamethasone, PGF $_{2\alpha}$ and estradiol. Cows carrying an *in vivo*-fertilized fetus were induced to undergo parturition in the same manner as the Clone group (DEX) or with PGF $_{2\alpha}$ and estradiol (PG), or the calves were delivered spontaneously (SP).

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-R α* 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 $F_{2\alpha}$ 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

Fig. 2. Correlation between the maternal plasma glucose concentration in the prepartum period and birth weight of clones derived from somatic cell nuclear transfer. Plasma glucose concentrations in cows carrying a somatic cell clone (●) or a control fetus (△) were measured at days 257 (A) and 271 (B) of gestation and at term (C).

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-R α* mRNA abundance, except for a decrease in *GC-R α* 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 placentome. 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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