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Endocrine Profiles and Blood Chemistry Patterns of Cloned Miniature Pigs in the Post-Puberty Period

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ABSTRACT

Although the majority of surviving pigs cloned by somatic cell nuclear transfer (SCNT) appear to be physiologically normal, there is a general lack of detailed hemato-physiologic studies for the period of early adulthood to substantiate this claim. In the present study, we investigated variation in blood chemistry and endocrinological parameters between mesenchymal stem cells (MSCs) derived from cloned and normal age-matched female and male miniature pigs. Cloned females and males showed normal ranges for complete blood count assessments. Biochemical assessments showed that γ-GGT, ALT and cholesterol levels of male and female clones were significantly ($P<0.05$ or $P<0.01$, respectively) higher than that of age-matched control miniature pigs. Variations in insulin and IGF-1 were higher in female clones than in male clones and controls. Thus, although female and male cloned miniature pigs may be physiologically similar to normal animals, or at least within normal ranges, a greater degree of physiological and endocrinological variation was found in cloned pigs. The above variation must be taken into account before considering cloned female or male miniature pigs for various biomedical applications.

(Key works : endocrine profile, blood chemistry, post-puberty, cloned animal, miniature pig)

INTRODUCTION

Therapeutic cloning by somatic cell nuclear transfer (SCNT) has the potential to assist in providing repaired cells, tissues, and organs for patients requiring supplementation because of incurable disorders or injury. Several studies have reported that cloned miniature pig are suitable as a donor animal model for tissue or organ xenotransplantation of human (Logan, 2000) because miniature pigs are superior in terms of similarities in gross anatomy, physiology and pathophysiology to humans as well as in terms of critical ethical problems. However, limited research has been performed with the overall information on the physiological health status and the endocrinological parameters in adult cloned miniature pigs.

Most animal cloning studies have been performed to enhance cloning efficiency by SCNT, and they have investigated genetic or physiological abnormalities in newborn to weaned cloned animals. Various animals have successfully been cloned by SCNT and several reports have conducted intensive follow-up studies of cloned animals to evaluate their epigenetic, behavioral and physiological status by SCNT (Chavatte-Palmer *et al*., 2002; Heyman *et al*., 2002; Archer *et al*., 2003; Dindot *et al*., 2004; Watanabe *et al*., 2008). However, the majority of studies were performed using observations of the physiological health of cloned cows (Lanza *et al*., 2001; Cibelli *et al*., 2002), or focused on global methylation differences, abnormal gene expression profiles (Ohgane J *et al*., 2001; Humpherys *et al*., 2002), and early death (Renard *et al*., 1999) of cloned mice.

Comprehensive studies on hematological and endocrinological variation of cloned miniature pigs after sexual maturation are lacking, although the majority of tissues or organs should be taken from adult donors for xenotransplantation. Moreover, prolonged gestation is common in clones, and live cloned offspring occasionally exhibit several types of abnormalities that may reduce their survival, and that may result in organ disorders or hemato-physiological problems in adulthood.

In the present study, we evaluated the hematological and biochemical status and endocrinological variation of cloned miniature pigs derived from bone marrow mesenchymal stem cells (BM-MSCs). In addition, gender-dependent hematological and physiological variation was observed between adult cloned miniature pigs and age- and gender-matched controls.

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MATERIALS AND METHODS

1. Animals

Cloned female and male miniature pigs generated from the same established line (Lee *et al*., 2010) were used in this study. Briefly, one-year-old male and female cloned miniature pigs were produced by SCNT using MSCs derived from BM-MSCs of miniature pigs. Three each of cloned female and male animals, and the same number of age- and gender-matched control animals were used in the study. All animals were maintained under identical conditions in a farm facility.

2. Blood Sample Collection

Blood samples were collected via jugular venipuncture 3 times at 3-month intervals to serve as a baseline for assessment values, especially endocrine values. Blood samples were collected at the same time of day (11:00 am). For blood chemistry and endocrinological assessment, the blood samples were centrifuged and the separated serum was stored at −20℃ until further analysis. For analysis of complete blood counts (CBC), blood samples were collected into EDTA-containing tubes.

3. Measurements and Assays

CBC analysis of leukocytes, erythrocytes and thrombocytes was performed using an automated hematology cell counter (MS9-5V; Melet Schloesing Lab, France). Briefly, differential blood cell counts were automatically performed per 100 cells.

Blood chemistry analyses were performed using a bench-top dry chemistry analyzer (Vettest 8008 Chemistry Analyzer; IDEXX Lab, UK) for the assessment of kidney, liver, and heart functions by examining creatinine, glucose, blood urea nitrogen (BUN), gamma-glutamyl transferase (γ-GGT), albumin, total bilirubin, total protein (TP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase, cholesterol, and amylase.

Assessments of endocrinological values were performed using a 7020 automatic analyzer (Hitachi, Japan) for the determination of plasma growth hormone (GH), insulin, insulin-like growth factor-1 (IGF-1), thyroid (T3), thyroxine (T4), cortisol, aldosterone, progesterone, testosterone, and estrogen levels.

4. Statistical Analysis

All data were analyzed by one-way analysis of variance (ANOVA; via SPSS). Data are expressed as means ± standard error of the mean (SEM), and differences were considered significant when $P<0.05$ or $P<0.01$.

RESULTS

Age and sex-matched cloned and control miniature pigs were successfully maintained in a healthy condition, without any disease during the duration of the experiments. CBC analysis of leukocytes, erythrocytes and thrombocytes was used to measure various parameters such as white blood cells (WBC), lymphocytes, monocytes, eosinophils, basophils, red blood cells (RBC), hematocrit, hemoglobin, uninfected RBC, macro RBC, total platelet count, mean platelet volume and plateletcrit from the collected whole blood samples 3 times at 3-month intervals (Table 1 and 2). CBC values were similar between cloned pigs and control pigs. The WBC levels were higher in cloned females and males than in controls; however, the observed levels are within the standard range for domestic pigs.

Cloned and control miniature pigs in the period post-puberty period showed some clinically significant differences between females and males serum chemistry parameters, or indicators of serum proteins, metabolic status and heart, liver and kidney function, such as creatinine, glucose, BUN, γ-GGT, albumin, total bilirubin, TP, ALT, AST, creatine kinase, cholesterol and amylase (Fig. 1). We monitored blood chemistry parameters at 3 time points for 9 months, and the majority of the parameters related to the biochemical status of clones were similar to those of control animals. However, in cloned males, the γ-GGT (67.0 ± 20.8) and ALT (78.7 ± 24.0) levels were above the normal range (16～30 and 9～43 U/l, respectively), and significantly ($P<0.05$ and $P<0.01$, respectively) higher than cloned females (γ-GGT; 38.7 ± 2.9, ALT; 55.0 ± 16.1) and control female and male pigs (γ-GGT : 27.5 ± 4.8 and 23.0 ± 4.4, ALT; 38.5 ± 7.9 and 32.3 ± 8.5). Cholesterol (85.0 ± 8.2 and 87.33 ± 6.7, respectively) levels of cloned males and females were above the normal range (18～79 mg/dl), and significantly ($P<0.05$) higher than control females and males (62.8 ± 3.6 and 57.0 ± 14.4, respectively).

Variation in the endocrine levels of cloned and control animals was monitored 3 times for 9 months, and results for GH, T3, T4, cortisol, aldosterone, progesterone, testosterone and estrogen levels were similar between gender-matched animals (Table 3 and 4). However, endocrinological variation in insulin and IGF-1 were higher in cloned females than in

Table 1. Mean values of complete blood counts for cloned and control female miniature pigs

Parameters (unit)	Cloned female mean	Control female	
		Mean	Range
WBC (m/mm^3)	17.04 ± 2.91	13.85 ± 0.71	13.05 ~ 14.41
Lymphocyte (%)	51.65 ± 8.08	53.33 ± 11.04	40.80 ~ 61.6
Monocyte (%)	7.65 ± 1.99	7.47 ± 1.15	6.80 ~ 8.8
Eosinophils (%)	2.25 ± 1.56	4.57 ± 3.33	2.40 ~ 8.4
Basophil (%)	0.25 ± 0.19	0.37 ± 0.25	0.10 ~ 0.6
RBC (M/mm^3)	7.94 ± 1.14	6.73 ± 1.50	5.49 ~ 8.4
Hematocrit (%)	43.83 ± 4.42	44.93 ± 1.59	43.10 ~ 45.9
Hemoglobin (g/dl)	14.20 ± 2.67	15.37 ± 2.00	13.10 ~ 16.9
Uninfected RBC (%)	9.10 ± 15.74	3.63 ± 5.08	0.60 ~ 9.5
Macro RBC (%)	5.68 ± 3.78	7.17 ± 6.18	0.30 ~ 12.3
Total platelet count ($10^3/\mu l$)	300.50 ± 100.77	359.00 ± 193.87	265 ~ 541
Mean platelet volume (fl)	7.75 ± 1.06	8.27 ± 2.80	6.50 ~ 11.5
Plateletcrit (%)	0.23 ± 0.13	0.30 ± 0.26	0.10 ~ 0.6

Values are expressed as mean ± SEM, Each group contains 3 animals.

Table 2. Mean values of complete blood counts for cloned and control male miniature pigs

Parameters (unit)	Cloned male mean	Control male	
		Mean	Range
WBC (m/mm^3)	15.68 ± 0.08	11.59 ± 5.96	5.55 ~ 17.46
Lymphocyte (%)	50.77 ± 5.44	48.07 ± 8.26	41.70 ~ 57.40
Monocyte (%)	9.43 ± 0.64	8.40 ± 2.91	5.60 ~ 11.40
Eosinophils (%)	1.10 ± 1.91	3.90 ± 2.07	1.70 ~ 5.80
Basophil (%)	0.47 ± 0.21	0.33 ± 0.21	0.10 ~ 0.50
RBC (M/mm^3)	7.15 ± 0.59	6.04 ± 2.38	3.35 ~ 7.86
Hematocrit (%)	39.00 ± 1.39	35.00 ± 10.69	22.70 ~ 42.00
Hemoglobin (g/dl)	13.50 ± 0.17	12.40 ± 4.78	6.90 ~ 15.60
Uninfected RBC (%)	7.80 ± 6.41	4.43 ± 6.55	0.60 ~ 12.00
Macro RBC (%)	4.00 ± 4.00	7.23 ± 5.66	0.70 ~ 10.30
Total platelet count ($10^3/\mu l$)	400.00 ± 131.64	306.00 ± 150.65	226 ~ 427
Mean platelet volume (fl)	8.13 ± 0.98	7.63 ± 2.12	5.70 ~ 9.90
Plateletcrit (%)	0.33 ± 0.12	0.23 ± 0.15	0.10 ~ 0.40

Values are expressed as mean ± SEM, Each group contains 3 animals.

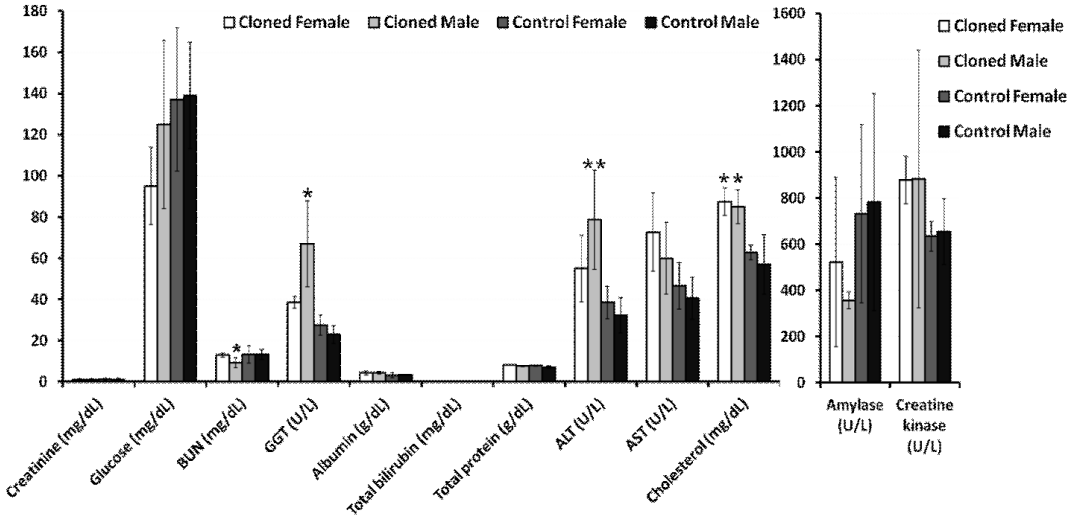

Fig. 1. Mean values of blood chemistry parameters of cloned and control miniature pigs. Data are expressed as the mean ± SEM of 3 pigs. Each group contains 3 animals. Different letters indicate significant differences (* P<0.05 or ** P<0.01). BUN, blood urea nitrogen; γ-GGT, gamma-glutamyltransferase; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

cloned males and controls.

DISCUSSION

Miniature pigs continue to elicit interest as an animal model in the fields of biomedical research (Polejaeva *et al*., 2000) and disease models for humans (Larsen *et al*., 2004) because of their suitability as donor animals for xenotransplantation (Xu *et al*., 2003). Hence, systematic studies of adult cloned miniature pigs may play an important role in various applications, including the safety of organ or tissue transplants from cloned miniature pigs and their offspring. However, information on the physiological status and endocrinological parameters of cloned miniature pigs is limited, not only for adults but also for prepubescent piglets.

Variation in biochemical and endocrinological parameters of physiologically and reproductively mature cloned miniature pigs

Table 3. Mean concentration of endocrinological parameters of cloned and control female miniature pigs

Parameters (unit)	Cloned female mean	Control female	
		Mean	Range
GH (ng/ml)	0.01 ± 0.01	0.03 ± 0.04	0.01 ~ 0.08
Insulin (uIU/ml)	1.43 ± 0.65	0.46 ± 0.34	0.20 ~ 1.00
IGF-1 (ng/ml)	226.10 ± 64.95	199.93 ± 8.90	191.60 ~ 209.30
T3 (ng/dl)	83.17 ± 15.33	78.53 ± 8.34	71.40 ~ 87.70
T4 (ng/dl)	3.55 ± 1.97	3.10 ± 1.86	1.86 ~ 5.24
Cortisol (ng/dl)	8.63 ± 2.49	8.61 ± 2.57	6.33 ~ 11.40
Aldosterone (pg/ml)	53.47 ± 63.43	68.87 ± 34.20	33.60 ~ 101.90
Progesterone (ng/ml)	20.98 ± 32.61	20.89 ± 33.87	1.08 ~ 60.00
Testosterone (ng/ml)	5.78 ± 4.71	3.89 ± 3.87	0.51 ~ 8.11
Estrogen (pg/ml)	2,513.67 ± 2,386.68	2,495.67 ± 2,394.02	229.80 ~ 5,000.00

Data are expressed as the mean ± SEM of 3pigs. Each group contains 3 animals.
GH, growth hormone and IGF-1, insulin like growth factor 1.

Table 4. Mean concentration of endocrinological parameters of cloned and control male miniature pigs

Parameters (unit)	Cloned male mean	Control male	
		Mean	Range
GH (ng/ml)	0.02 ± 0.02	0.03 ± 0.03	0.01 ~ 0.07
Insulin (uIU/ml)	0.93 ± 0.12	0.48 ± 0.46	0.20 ~ 1.00
IGF-1 (ng/ml)	174.17 ± 42.16	168.97 ± 21.17	146.90 ~ 189.10
T3 (ng/dl)	71.33 ± 18.57	64.40 ± 22.62	50.40 ~ 90.50
T4 (ng/dl)	3.10 ± 0.68	3.02 ± 0.23	2.79 ~ 3.24
Cortisol (ng/dl)	9.15 ± 5.13	6.79 ± 3.52	3.72 ~ 10.63
Aldosterone (pg/ml)	44.50 ± 39.12	54.33 ± 11.11	41.90 ~ 57.80
Progesterone (ng/ml)	38.22 ± 32.55	32.95 ± 27.18	3.31 ~ 56.70
Testosterone (ng/ml)	2.19 ± 3.30	2.82 ± 4.60	0.16 ~ 8.14
Estrogen (pg/ml)	1,445.33 ± 2,390.19	1,215.57 ± 1,161.89	25.10 ~ 2,346.6

Data are expressed as the mean ± SEM of 3 pigs. Each group contains 3 animals.
GH, growth hormone and IGF-1, insulin like growth factor 1.

should be examined to determine the probable adaptive function or potential side effects of transplanted cells or organs derived from cloned animals. To clarify these issues, we compared gender-dependent differences in hematological, biochemical and endocrinological variation to identify organ function and the physiological status of adult cloned miniature pigs.

As shown in Table 1, mean WBC values of cloned pigs tended to be higher than controls, although hematological values remained within normal ranges in all cloned pigs, similar to controls. Thus, we confirmed that all animals retained their healthy status under normal conditions, without any infection or disease.

The most common mortality factors in cloned cattle, representing 24% of deaths in the period between weaning and adulthood, are related to abnormalities of the musculoskeletal system. Mir (Mir *et al*., 2005) compared blood profiles between cloned pigs, control pigs, and their progeny to quantify the effect of cloning on several blood parameters and their transmission to the next generation; the variability in all traits in F_1 progeny of clones and the control pigs was similar, with the exception of BUN and ALP. Thus, although cloned animals may be physiologically normal or at least within normal ranges, periodic disorders may still occur in adulthood that require treatment. Moreover, unexpected health complications may not be observed in clones until the animals are stressed or aged. In the present study, the variability in biochemical parameters such as creatinine, glucose, BUN, γ-GGT, albumin, TP, AST, creatine kinase, cholesterol and amylase, observed in cloned fe- males and males was similar to that in controls. However, mean values of γ-GGT and ALT were significantly (P<0.05 and P<0.01, respectively) higher in cloned males than in cloned females and control females and males. Mean values of cholesterol were significantly (P< 0.05) higher in cloned females and males than in control females and males. Values of γ-GGT are the functional parameter for the kidney, and values of ALT is one of the functional parameters of the liver. Hence, our results suggest that some parameters of liver and kidney function differed significantly between controls and cloned males and females.

Measurements of endocrinological variation are important to fully understand the physiological status of cloned animals during adulthood. The endocrine profiles of cloned goats have been reported to show greater variability than that of controls for GH, T3 and insulin (Landry *et al*., 2005). In the present study, we found that insulin and IGF-1 levels were higher in cloned females than in cloned males and controls.

Variation in T3, T4 and insulin levels has been reported in cloned animals; insulin, T3 and T4 concentrations of cloned calves differed from controls, measured 10 min following birth (Garry *et al*. 1996), and T4 and insulin levels differ between cloned cows and age- and weight-matched controls (Kyle *et al*., 2002). The present study indicates that while cloned miniature

pigs may be within the normal range for the majority of CBC, biochemical and endocrinological parameters, they do exhibit significant differences in some of parameters of kidney and liver functions, and insulin and IGF-1. We suggest that these results could provide useful hematological and physiological parameters for cloned miniature pigs and normal miniature pigs, although these findings may be due to the effects of micro-manipulation in the *in vitro* culture environment or post-implantation environmental effects.

CONCLUSION

In conclusion, we found a measurable degree of biochemical and endocrinological variation in cloned miniature pigs derived from BM-MSCs, despite similarities in hematological and biochemical parameters between cloned male and female miniature pigs and controls. This variation must be taken into account before considering the cloned miniature pigs for application in biomedicine and cell or organ xenotransplantation.

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