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Analysis of Semen Parameters in α1,3-Galactosyltransferase$^{-/-}$ Boars

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ABSTRACT

It is very difficult to get the information about semen quality analysis in transgenic pigs because of limited numbers and research facilities. Therefore, in the present study, we analyzed the semen quality of transgenic boars generated for xenotransplantation research. Briefly, the semen samples were collected from 5 homozygous *α*1,3-Galactosyltransferase knock-out (GalT$^{-/-}$) transgenic boars and immediately transported to the laboratory. These semen samples were decupled with DPBS and conducted to analyze semen parameters by a computer-assisted semen analysis (CASA) system. The boar semen were examined all 12 parameters such as total motility (TM), curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), and hyperactivated (HYP), etc. In results, among the 5 GalT$^{-/-}$ boars, three boars (#134, 144, and 170) showed normal range of semen parameters, but #199 and 171 boars showed abnormal ranges of semen parameters according to standard ranges of semen parameters. Unfortunately, #171 boar showed azoospermia symptom with rare sperm counts in the original semen. Conclusively, assessment of semen parameters by CASA system is useful to pre-screening of reproductively healthy boar prior to natural mating and artificial insemination for multiplication and breeding.

(Key words: Semen parameters, CASA, Transgenic boars, Xenotransplantation)

INTRODUCTION

Xenotransplantation of pig organs into human has been considered as a promising solution to the critical shortage of organs from deceased human donors for transplantation (Abouna 2008). However, the immune rejection response caused by species differences between pigs and human are the main obstacle for successful xenotransplantation (Kwon et al. 2016, Yamada et al. 2005). To overcome this problem, many research groups are still striving to produce transgenic pig with controlled immunodeficiency, including knockout of the α-1,3-galactosyltransferase (GalT) gene for the deletion of galactose-α-1,3-galactose antigens and the knock-in of human complement-regulatory genes for inhibition of human complement activation, such as CD46, CD55 and CD59 (Kwon et al. 2016, Lai et al. 2002, Lee et al. 2011). On the other hand, the reproductive disorder in transgenic pigs has been observed occasionally including irregular estrus (Lee et al. 2008), testicular abnormality (Choi et al. 2012), and decreased sperm fertility (Park et al. 2006) even though we reported the production of the heterozygous GalT (GalT$^{-/+}$) pigs with normal reproductive ability (Hwang et al. 2012).

For diagnosis of reproductive disorder, one possible method is direct analysis of semen quality and motility through the use of computer-assisted semen analysis (CASA) system (Amann and Waberski 2014). Although the relationship between semen parameters and fertilization ability has not yet been analyzed well, the CASA system is the most popular and reliable method used to evaluate sperm quality and motility (Gil et al. 2009, Verstegen et al. 2002). Additionally, many studies have been described the possible correlations of sperm motility and fertility from many species including cattle (Budworth et al. 1988), horse (Samper et al. 1991), humans (Hirano et al. 2001), rabbits (Lavara et al. 2005), and pigs (Vyt et al. 2008).

Therefore, in the present study, we conducted to investigate the sperm quality and motility by CASA system to diagnose

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whether the GalT$^{-/-}$ pigs are reproductively healthy or not. Additionally, the semen parameters were categorized into three parts based on total motility, velocity, and movement of sperm.

MATERIALS and METHODS

1. General information

All chemicals used in the present study were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated. Also, our study protocol and standard operating procedures for the treatments of the pigs used present study were reviewed and approved by the Institutional Animal Care and Use Committee of the National Institute of Animal Science, RDA.

2. Production and breeding of GalT$^{-/-}$ boars

Production of GalT$^{-/+}$ transgenic cloned pigs by somatic cell nuclear transfer was performed as previous study reported by Hwang et al. (2013). Briefly, ovaries were obtained from local slaughterhouse (Nonghyup Moguchon, Gimje, Korea), and the oocytes were matured *in vitro*. The cloned transgenic embryos were transferred into both oviducts of the surrogate (Landrace) on the same day or 1 day after the onset of estrus. After gestation period, the transgenic cloned piglets (Chicago minipigs) were delivered by natural parturition or Caesarean section. Using the GalT$^{-/+}$ transgenic cloned pigs, GalT$^{-/-}$ pigs were generated by breeding.

3. Semen collection and CASA analysis

Semen samples from 5 GalT$^{-/-}$ transgenic boars (36±5 months and 86±6 kg) were collected once a week by conventional globed-hand method. Briefly, each ejaculate was collected within a paper cup (350 ml-sized) covered with sterilized gauze to remove the gel fraction. Freshly ejaculated semen samples were transported to laboratory immediately and decupled with DPBS. Then, the semen samples were conducted to analyze sperm quality with CASA system (Medical Supply Co. Ltd, Korea). The total motility scores of each semen samples were obtained on a routine basis using a CASA system attached to a microscope (Olympus, Japan) in a laboratory at room temperature (25±5 °C). Approximately 5 μl of decupled semen samples was mounted on a MAKLER counting chamber (Irvine Scientific, USA) placed on warm plate (Kitazato, Japan). All semen parameters and their relationships were recorded by CASA system which is described minutely in Figure 1. As described in Figure 1, the semen parameters were categorized into three parts based on total motility (TM), velocity (VCL, VSL, VAP, and velocity distribution), and movement of sperm (LIN, ALH, HYP, STR, BCF, MAD, and WOB). Normal range and unit of semen parameters were also indicated in Figure 1.

4. Statistics

All semen parameters were analyzed 5 times in individual semen samples. Using the datasets recorded by CASA system, the significant differences were analyzed by the Student's *t* test. A value of $P < 0.05$ was considered to be a significant difference.

RESULTS

1. Semen morphology and total motility analysis of sperm from individual transgenic boars

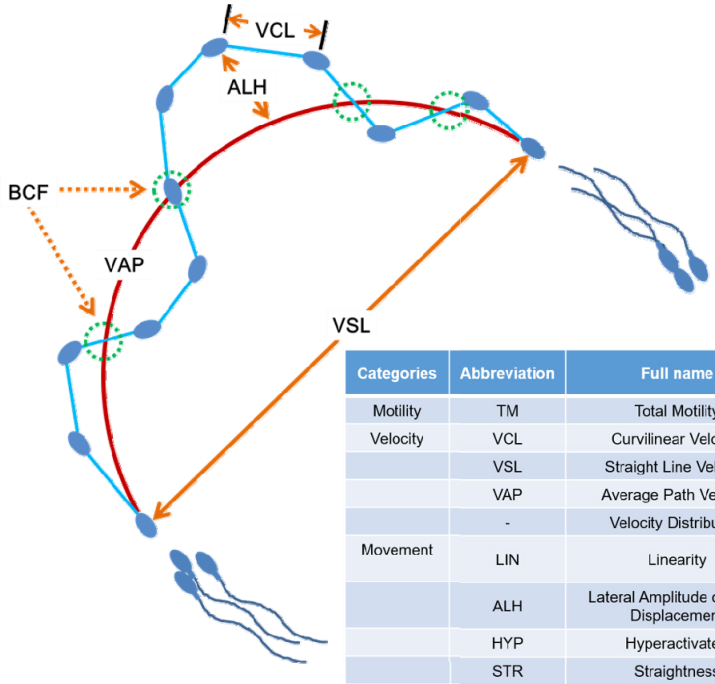
Freshly ejaculated semen samples were decupled with DPBS prior to analysis of morphological abnormality and motility by

Table 1. Analysis of movement-related semen parameters in individual transgenic boar.

Individual IDs	Movement-related sperm parameter of *						
	LIN (%)	ALH (μm)	HYP (%)	STR (%)	BCF (Hz)	MAD (º)	WOB (%)
#134	43.3±1.3^{a}	4.0±0.1^{a}	49.4±2.9^{a}	53.8±1.4^{a}	5.1±0.2^{a}	9.6±0.3^{a}	70.9±1.1^{a}
#144	39.4±0.9^{b}	4.5±0.1^{b}	61.0±1.7^{b}	50.4±0.8^{a}	5.7±0.2^{b}	8.4±0.5^{a}	70.6±1.0^{a}
#170	39.7±1.0^{b}	4.6±0.1^{b}	61.7±2.2^{b}	51.0±0.6^{a}	6.0±0.1^{b}	9.4±0.5^{a}	71.2±0.8^{a}
#171	68.2±11.0^{c}	1.3±0.3^{c}	3.6±0.5^{c}	77.8±10.7^{b}	1.0±0.3^{c}	2.2±0.1^{b}	87.8±5.0^{b}
#199	48.1±1.4^{d}	2.1±0.0^{d}	7.6±2.5^{c}	47.2±7.9^{a}	2.4±0.4^{d}	1.8±0.4^{b}	78.2±2.5^{b}

*Data are expressed as Mean±SEM of five replicates in each individual transgenic boar. a-d Different superscripts denote significant differences within column ($P < 0.05$). Full name of semen parameters are indicated in Figure 1.

CASA system. A semen sample from transgenic boar #171 showed that the number of sperm was very limited and very high abnormality was observed (data not shown). Thus the semen sample from #171 was conducted following experiment and analysis with original fresh semen without dilution. The morphological abnormality was not found from transgenic boars except transgenic boar #171. As shown in Figure 2, the total motility of transgenic boars #134 (85.2 %), #144 (85.6 %), and #170 (87.9 %) were significantly (P<0.05) higher than that of transgenic boars #171 (6.1 %) and #199 (21.2 %).



Categories	Abbreviation	Full name	Normal range and unit
Motility	TM	Total Motility	>> 50 %
Velocity	VCL	Curvilinear Velocity	>> 46 μm/s
	VSL	Straight Line Velocity	>> 26 μm/s
	VAP	Average Path Velocity	μm/s
	-	Velocity Distribution	Each %
Movement	LIN	Linearity	>> 58, VSL / VCL
	ALH	Lateral Amplitude of Head Displacement	>> 2.4 μm
	HYP	Hyperactivated	%
	STR	Straightness	VSL / VAP
	BCF	Beat Cross Frequency	Hz
	MAD	Mean Angular Displacement	Degree
	WOB	Wobble	VAP / VCL

Figure 1 Terminology of semen parameters by computer-assisted sperm analysis (CASA) system.
These semen parameters were divided into three groups based on total motility (TM), velocity (VCL, VSL, VAP, and velocity distribution), and movement of sperm (LIN, ALH, HYP, STR, BCF, MAD, and WOB).

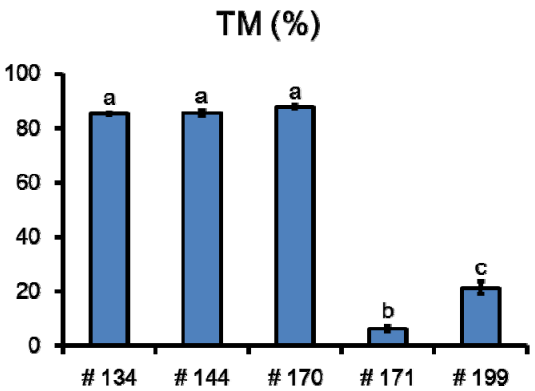


Figure 2 Analysis of total motility in individual GalT$^{-/-}$ transgenic boars.
The values of TM for each boar were produced by CASA system automatically. $^{a-c}$ Different superscripts denote significant differences (P<0.05). TM, total motility.

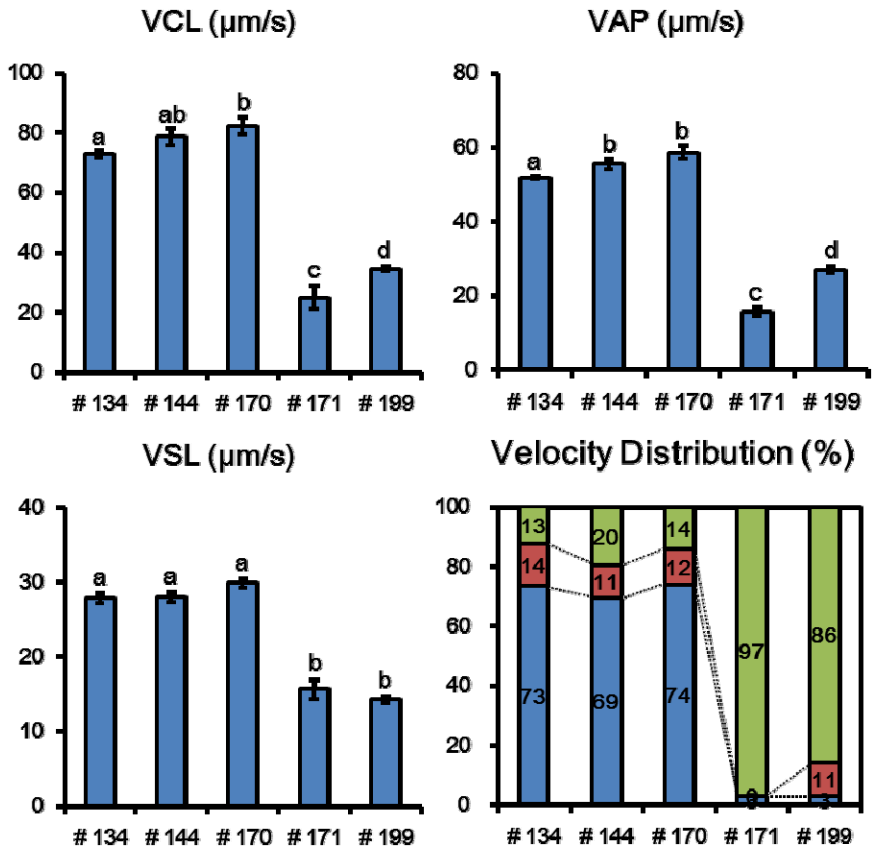


Figure 3 Analysis of velocity-related sperm parameters in individual GalT$^{-/-}$ transgenic boars.
The values of VCL, VAP, and VSL for each boar were produced by CASA system automatically. In velocity distribution histogram, the green, red, and blue bars indicate velocity distribution of slow (1-49 μm/s), medium (50-99 μm/s) and rapid (>100 μm/s), respectively. $^{a-d}$ Different superscripts denote significant differences (P<0.05). VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity.

2. Velocity analysis of sperm from individual transgenic boars

Results of sperm velocity and velocity distribution are represented in Figure 3. Firstly, the value of VCL in transgenic boars #134 (72.7 %), #144 (78.6 %), and #170 (82.3 %) were significantly (P<0.05) higher than that of transgenic boars #171 (25.0 %) and #199 (34.5 %). Secondly, the value of VSL in transgenic boars #134 (27.8 %), #144 (28.0 %), and #170 (29.8 %) were significantly (P<0.05) higher than that of transgenic boars #171 (15.6 %) and #199 (14.3 %). Thirdly, the value of VAP in transgenic boars #134 (51.6 %), #144 (55.5 %), and #170 (58.6 %) were significantly (P<0.05) higher than that of transgenic boars #171 (15.6 %) and #199 (26.9 %). Finally, the portion of rapid sperm was significantly (P<0.05) in transgenic boar #134 (73.4 %), #144 (69.3 %), and #170 (73.9 %) than #171 (3.0 %) and #199 (2.8 %). On the other hand, the portion of slow sperm was significantly (P<0.05) higher in transgenic boar #171 (97.0 %) and #199 (85.9%) than #134 (12.7 %), #144 (19.7 %), and #170 (14.2 %). Additionally, the portion of medium sperm in transgenic boar # 171 was not observed while other transgenic boars showed comparable (#134, 14.0 %; #144, 11.0 %; #170, 11.9 %; #199, 11.3 %).

3. Movement analysis of sperm from individual transgenic boars

Results of sperm movement analysis are represented in Table 1. Among the 5 transgenic boars, three (#134, 144, and 170) showed normal range in 7 criteria (LIN, ALH, HYP, STR, BCF, MAD, and WOB) for sperm movement, but #199 and #171 boar showed significantly (P<0.05) abnormal range in 7 criteria compared with normal three transgenic boars (#134, 144, and 170).

DISCUSSION

Conventional methods for analyzing sperm quality, such as morphology, motility, and concentration or volume, allow researcher to judge an optimal qualification of boar for breeding and multiplication by artificial insemination (Jung et al. 2015). The CASA system also allows researcher to judge a best candidate for breeding and multiplication by artificial insemination. However, not all of these standard sperm parameters are directly related to boar fertility (Berger et al. 1996). Because, fertilization procedures by artificial insemination are very complex process involving a large number of events occurred in the female reproductive tract between sperm and oocyte.

When the GalT$^{-/+}$ produced for the first time, we tried to multiply the number of transgenic pigs by artificial insemination and natural mating. The GalT$^{-/+}$ founder successfully produced the next generation and reproductive disorder was not observed during this period (Hwang et al. 2012). Then the GalT$^{-/-}$ were generated successfully from the GalT$^{-/+}$ pigs. In the present study, we analyzed semen quality of GalT$^{-/-}$ by the CASA system because, we found that some of GalT$^{-/-}$ showed reproductive disorders such as relatively low pregnancy rates and relatively small litter size. According to recent reports, the reproductive disorder in transgenic pig has been occurred occasionally such as irregular estrus (Lee et al. 2008), testicular abnormality (Choi et al. 2012), and decreased sperm fertility (Park et al. 2006). In the present study, two fifths of transgenic boars showed decreased total motility, reduced sperm velocity, and abnormal movement among 5 transgenic boars. Additionally, the first GalT$^{-/-}$ pig was developed more than a decade ago, there is still no enough report about reproductive disorder. Therefore, genetic and physiological analysis of GalT$^{-/-}$ will be required to confirm this phenomenon.

Regarding the relationship between sperm motility and fertility, many studies has been reported. Flowers (1997) reported that no relationship between the sperm motility and fertility was founded in vitro as well as in vivo when the sperm motility was more than 60 %. However, the sperm motility was less than 60 %, fertility was reduced (Flowers 1997). In the present study we also found that boars with very low (less than 20 %) sperm motility showed infertility symptom. Additionally, the kinetic parameters such as VAP, VSL, VCL, STR and BCF of spermatozoa after 2-h incubation at 39°C were significant predictors of litter size (Holt et al. 1997).

Conclusively, we reported the symptom of infertility and/or reproductive disorder in GalT$^{-/-}$ for the first time by analysis of semen with CASA system. Further studies are still needed to be clarified the problems related to reproductive disorder in GalT$^{-/-}$ pigs by genetic and physiological analysis.

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