Sperm Characteristics in Microminipigs

TATSUO KAWARASAKI¹, SATOKO ENYA² and MASAYOSHI OTAKE²

¹*School of Agriculture, Tokai University, Kumamoto, Japan;*

²*Swine and Poultry Department, Swine and Poultry Research Center,
Shizuoka Prefectural Research Institute of Animal Industry, Shizuoka, Japan*

Abstract. *Background/Aim: The microminipig is a relatively new type of mini pig; microminipigs weigh about 10 kg at 6 months of age and are expected to be of use in drug discovery research and safety tests. Herein, we analyzed the characteristics of ejaculated sperm from microminipigs. Materials and Methods: Sperm parameters such as microstructure and sensitivity to cold shock were investigated using optical or scanning electron microscopy. Results: Ejaculate volumes and total numbers of sperm were lower than in standard pig strains, but were proportional to body weight. Ejaculation time, pH of the ejaculate, sperm motility and morphology, and sensitivity to cold shock were similar to those of standard pig strains. Conclusion: Herein, we provide the first characterization of the ejaculates of microminipigs and demonstrate that this type of pig will be useful not only in medical research, but also in investigations into sperm preservation in different pig breeds.*

The microminipig is a type of experimental mini pig that was developed by crossing pot-bellied pigs with other strains of mini pigs (1). Microminipigs weigh approximately 5 kg at 3 months of age and less than 10 kg at 6 months of age; hematological and blood biochemical values are similar to those of Göttingen mini pigs and Yucatan mini pigs, and show no differences between males and females (2). Genomic analyses have shown that microminipigs show no significant differences to standard pig strains (3). The swine leukocyte antigen (SLA) haplotypes of the basal generation pigs have been identified (4, 5). Microminipigs have a similar lipid

metabolism profile as humans with regard to low-density lipoprotein cholesterol (LDL-C) (6); they also display hyperlipidemia when fed a high-fat, high-cholesterol diet, and atherosclerosis (7) and liver cancer (8) can be induced. Microminipigs have also been used in pharmacological studies (9-16) and in regenerative medicine (17-19).

Microminipig females reach puberty at 5 months of age and sexual maturity at 8 months of age similarly to standard domestic pigs (20). They also show the same estrous characteristics as standard pigs and other miniature pig strains, with similar gonadotropin and ovarian steroid profiles throughout the estrous cycle (21). Estrogen treatment can be used to induce pseudopregnancy in microminipig females, and subsequent prostaglandin treatment allows synchronization of estrus (22, 23). Male microminipigs reach sexual maturity at around 4.5 months of age as shown by analysis of testicular tissues and epididymides, and by growth of accessory gonadal tissue (24). The duration of spermatogenesis is similar to that of domestic pig breeds (25).

Although some aspects of the reproductive characteristics of microminipigs have been determined, information on ejaculated sperm is scarce. This information is of importance for use of microminipigs in reproductive technologies such as artificial insemination, *in vitro* insemination, and cryopreservation of spermatozoa. The present study was initiated to remedy these deficits in our knowledge by an in-depth analysis of microminipig sperm; the general properties of ejaculated sperm, the susceptibility of sperm to cold shock, and the morphology of spermatozoa were examined by optical and scanning electron microscopy.

Materials and Methods

Examination of sperm parameter. Five mature male microminipigs (Fujimicra, Inc., Shizuoka, Japan) aged 23.4±1.1 months (mean±SE) and weight 19.7±1.1 kg (mean±SE) were used for analysis of sperm characteristics. The microminipigs were kept in individual cages with water available *ad libitum* and were fed a commercial domestic pig diet (Marubeni Nisshin Feed Co. Tokyo, Japan). Animals were treated in accordance with the guidelines of Tokai University. All experimental protocols were approved by Tokai University (#155026).

This article is freely accessible online.

Correspondence to: Tatsuo Kawarasaki, DVM, Ph.D., Laboratory of Anima Reproduction, School of Agriculture, Tokai University, 9-1-1 Toroku, Higashi-ku, Kumamoto, 862-8652, Japan. Tel: +81 963862816, Fax: +81 963817956, e-mail: kawarasaki2016@gmail.com

Key Words: Pig, microminipig, sperm, characteristic, cold shock, morphology, SEM.

Sperm examination. Whole sperm was collected using the gloved hand method (26). Sperm quality was assessed from sperm volume, sperm concentration, sperm motility, and proportion of spermatozoa with normal morphology. The sperm examination methods were based on those described by Kawarasaki *et al.* (27). Briefly, two 1 ml samples were removed from each whole filtered ejaculate; one sample was held at 37°C for 15 min and then the proportion of spermatozoa with progressive motility, that is moving actively, either linearly or in a large circle regardless of speed, was estimated by phase-contrast microscopy. The other sample was fixed with 1% formalin and the morphology of the sperm was examined by phase-contrast microscopy (Nikon Corporation, Tokyo, Japan) to evaluate acrosome formation as described by Pursel and Johnson (28). A total of 200 spermatozoa were examined from each sample, and the proportion of morphologically normal spermatozoa was calculated. Sperm concentration was determined using a hemocytometer. The pH of each sperm sample was measured using a pH meter (Horiba, Ltd. Kyoto, Japan).

Scanning electron microscopy (SEM) analysis of spermatozoa. Five male microminipigs (29.0±12.2 months old), five male Landrace pigs (21.5±3.4 months old), and five male Duroc pigs (31.0±6.7 months old) were used for the SEM analysis. The management of the microminipigs was identical to that described above for analysis of sperm characteristics. The Landrace and Duroc pigs were bred at the Shizuoka Prefectural Research Institute and were kept in individual cages with water available ad libitum and were fed a commercial domestic pig diet (JA Higashinohon Kumiai Shiryō Co., Ltd, Oota, Japan). All procedures were approved by the Animal Care and Use Committee of Shizuoka Prefectural Research Institute of Animal Industry, Swine and Poultry Research Centre and complied with the Guidelines for Proper Conduct of Animal Experiments from the Science Council of Japan (29).

One ml of each sperm sample was centrifuged at 740×g for 5 min at room temperature. The supernatants were removed and pellets were resuspended and fixed in 2.5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2~7.4) for several days at 4°C (primary fixation). Subsequently, several drops of each sample were loaded onto a membrane filter in an SEM pore sample holder and fixed with 2.5% glutaraldehyde and 1% osmium acid. After dehydration through an ethanol series, the specimens were freeze-dried using t-butyl alcohol, coated with gold, and examined using a thermal electron gun type SEM (JSM-6510; JEOL Ltd., Tokyo, Japan). Ten spermatozoa from each male were analyzed by SEM. Measurement positions are shown in Figure 1.

Reaction to cold shock. Two mature male microminipigs (11.8 and 15.9 months old) were used for analysis of the effects of cold shock on spermatozoa. The management of the microminipigs was identical to that described above. Five ejaculates were collected from each male. An aliquot of each ejaculate was centrifuged at 1,700×g for 30 min at 4°C and the seminal plasma was collected. Within 30 min of collection, sperm aliquots were diluted 1:3, 1:7, or 1:11 with seminal plasma and Tris-lactose extender (30) so that each sample was composed of 50% Tris-lactose extender and 50% spermatozoa-seminal plasma. The diluted samples were incubated at 30°C for 1, 3, or 5 h after collection. One-milliliter samples in 16×100 mm screw-cap glass tubes were cold shocked by plunging into a 5°C water bath for 10 min, after which sperm motility and acrosome morphology were assessed.

Figure 1. Measurement positions in spermatozoa. The positions where the spermatozoa were measured are indicated by green or orange lines. Ten spermatozoa from each of six microminipigs, five Landrace pigs and five Duroc pigs were analyzed by SEM. Whole length (A①), head length (B②), head width (width1, widest part B③; width2, post acrosomal region B④), head thickness (thickness1, peri-acrosomal part C⑤; thickness 2, post-acrosomal region C⑥), neck length (B⑦), midpiece length (D⑧), midpiece width (E⑨), principal piece width (width1, near start point E⑩; width 2, near end point F⑪), end piece length (F⑫), and end piece widths (width1, narrow part F⑬; width2, thick part F⑭) were measured.

Statistical analysis. Data are presented as means±standard error (SE). Percentage data were arcsine-transformed before analysis. All data were analyzed by ANOVA using StatView software (Windows version 5; SAS Institute Inc., Cary, NC, USA).

Results

Sperm parameters of microminipigs. The sperm parameters (mean±SE) of ejaculated sperm are shown in Table I and Table II. Ejaculation time, sperm pH, total sperm count, and sperm concentration differed between individuals (ejaculation time, $p<0.0001$; pH, $p<0.0001$; total sperm count, $p=0.001$; sperm concentration, $p<0.0001$). A mean sperm volume of 63.1±2.0 ml was obtained, with no significant differences between individuals. The mean rate of sperm with progressive motility was 81.9±4.9%, with no significant differences between individuals. The sperm morphology parameters obtained in the optical microscope analysis are shown in Table II. The mean proportion of normal sperm was 89.3±1.0%; one individual had a significantly lower proportion of normal sperm ($p<0.0001$). Abnormal tail structure was the most common anomaly, and abnormalities of the head were comparatively infrequent. The mean rate of sperm with a normal acrosome was 92.5±1.3%; no significant differences were observed between individuals.

SEM analysis of spermatozoa. The results of the SEM analysis of sperm morphologies in microminipigs, Landrace, and Duroc pigs are presented in Table III and Table IV. The sperm

Table I. Sperm characteristics of microminipigs.

Male	Number of ejaculates	Age (months)	Time for ejaculation (sec.)	pH	Sperm volume (ml)	Concentration of spermatozoa/ml ($\times 10^8$)	Total no. of spermatozoa ($\times 10^8$)
M1	16	23.1 $\pm$ 1.1	275.6 $\pm$ 15.4 ^b	7.7 $\pm$ 0.1 ^{ab}	57.4 $\pm$ 7.6	0.996 $\pm$ 0.087 ^{ab}	52.7 $\pm$ 7.5 ^{bc}
M2	19	20.8 $\pm$ 0.9	326.7 $\pm$ 17.6	7.3 $\pm$ 0.0 ^d	68.6 $\pm$ 5.0	0.424 $\pm$ 0.045 ^e	29.6 $\pm$ 4.5 ^d
M3	18	26.0 $\pm$ 1.7	363.1 $\pm$ 18.7 ^a	7.5 $\pm$ 0.1 ^{bc}	61.7 $\pm$ 8.8	0.625 $\pm$ 0.082 ^{de}	31.7 $\pm$ 4.2 ^{cd}
M4	15	25.9 $\pm$ 2.0	265.3 $\pm$ 14.2 ^b	7.2 $\pm$ 0.1 ^{cd}	61.4 $\pm$ 7.9	0.930 $\pm$ 0.061 ^{bc}	59.1 $\pm$ 7.5 ^{ab}
M5	15	21.1 $\pm$ 1.7	258.0 $\pm$ 10.7 ^b	7.3 $\pm$ 0.1	66.5 $\pm$ 6.2	0.820 $\pm$ 0.860 ^{cd}	51.2 $\pm$ 6.0
Total		23.4 $\pm$ 1.1	297.7 $\pm$ 8.6	7.4 $\pm$ 0.1	63.1 $\pm$ 2.0	0.739 $\pm$ 0.040	44.8 $\pm$ 6.0

Values are mean $\pm$ SE. Different superscripts in same column indicate significant differences ($p < 0.05$).

Table II. Sperm motility and morphology in microminipigs.

Male	Number of ejaculates	Motile spermatozoa* (%)	Acrosomal integrity (%)	Morphology of spermatozoa (%)		
				Normal	Abnormal	
					Tail	Head
M1	16	87.5 $\pm$ 7.0	92.5 $\pm$ 1.7	93.6 $\pm$ 1.3 ^a	2.9 $\pm$ 0.4 ^b	2.4 $\pm$ 1.2
M2	19	93.2 $\pm$ 1.7	95.2 $\pm$ 0.6	92.0 $\pm$ 2.0 ^a	8.0 $\pm$ 2.0 ^b	0 $\pm$ 0
M3	18	85.3 $\pm$ 6.2	92.0 $\pm$ 1.8	91.9 $\pm$ 1.3 ^a	7.2 $\pm$ 1.4 ^b	0.9 $\pm$ 0.4
M4	15	79.3 $\pm$ 5.8	94.9 $\pm$ 1.6	90.1 $\pm$ 1.9 ^a	8.6 $\pm$ 1.9 ^b	1.3 $\pm$ 0.8
M5	15	64.3 $\pm$ 9.9	87.8 $\pm$ 3.5	78.2 $\pm$ 1.9 ^b	19.8 $\pm$ 2.1 ^a	2.0 $\pm$ 1.7
Average		81.9 $\pm$ 4.9	92.5 $\pm$ 1.3	89.3 $\pm$ 1.0	10.0 $\pm$ 1.0	1.3 $\pm$ 0.4

Values are mean $\pm$ SE. Different superscripts in same column indicate significant differences ($p < 0.05$). *Spermatozoa with progressive motility, that is moving actively, either linearly or in a large circle, regardless of speed.

Table III. Morphology of spermatozoa in microminipigs, Landrace and Duroc pigs.

Breed	No. of males	Sperm length	Head					Neck length
			Length	Width1	Width2	Thickness1	Thickness2	
Microminipig	5	57.28 $\pm$ 0.17 ^a	7.37 $\pm$ 0.06	4.17 $\pm$ 0.03 ^a	3.59 $\pm$ 0.03 ^a	0.52 $\pm$ 0.01 ^a	0.35 $\pm$ 0.01 ^a	1.42 $\pm$ 0.02 ^a
Landrace	5	56.19 $\pm$ 0.22 ^b	7.41 $\pm$ 0.06	3.95 $\pm$ 0.02 ^b	3.48 $\pm$ 0.02 ^b	0.45 $\pm$ 0.01 ^b	0.35 $\pm$ 0.01	1.30 $\pm$ 0.03 ^b
Duroc	5	53.82 $\pm$ 0.20 ^c	7.53 $\pm$ 0.04	3.92 $\pm$ 0.02 ^b	3.49 $\pm$ 0.03 ^b	0.44 $\pm$ 0.01 ^b	0.33 $\pm$ 0.00 ^b	1.26 $\pm$ 0.02 ^b

Measurement positions are shown in Figure 1. Values are mean $\pm$ SE. Different superscripts in same column indicate significant differences ($p < 0.05$).

of microminipigs were 1.2% longer than those of Landrace pigs and 6.4% longer than those of Duroc pigs; the differences among breeds were significant ($p < 0.0001$). There was no difference in mean head length among the three breeds, but the sperm of microminipigs had longer neck length, middle piece length, and main part length than the other two breeds ($p < 0.0001$). With regard to end piece length, that of Landrace sperm was longer than in the other two breeds ($p < 0.0001$).

Head width was larger in microminipig sperm than in Landrace or Duroc sperm ($p < 0.0001$). Head thickness, middle piece widths 1 and 2, and principal piece widths were

larger in microminipig sperm than in Landrace and/or Duroc sperm (Table III).

Reaction to cold shock. Sperm motility rates after cold shock varied with pre-culture time: higher rates were seen at longer times of culture and also at lower ratios of dilution (Figure 2). No interaction was observed between culture time and dilution rate. The proportion of sperm with normal acrosomes increased with culture time (Figure 3). After 5 h culture, the proportion of sperm with normal acrosomes was higher in samples with lower levels of dilution (Figure 3). No

Table IV. Morphology of spermatozoa in microminipigs, Landrace and Duroc pigs.

Breed	No. of males	Mid piece			Principal piece			End piece		
		Length	Width1	Width2	Length	Width1	Width2	Length	Width1	Width2
Microminipig	5	11.63±0.05 ^a	0.72±0.01 ^a	0.55±0.01 ^a	34.72±0.16 ^a	0.49±0.01 ^a	0.38±0.00 ^a	2.26±0.04 ^b	0.17±0.00 ^b	0.28±0.01 ^a
Landrace	5	11.23±0.05 ^b	0.73±0.01 ^a	0.56±0.01 ^a	33.68±0.18 ^b	0.49±0.00 ^a	0.35±0.01 ^b	2.59±0.06 ^a	0.19±0.01 ^a	0.26±0.01
Duroc	5	11.02±0.09 ^b	0.68±0.01 ^b	0.53±0.01 ^b	31.80±0.14 ^c	0.47±0.01 ^b	0.36±0.01 ^b	2.21±0.03 ^b	0.19±0.01	0.23±0.02 ^b

Measurement positions are shown in Figure 1. Values are mean±SE. Different superscripts in same column indicate significant differences ($p<0.05$).

Figure 2. Proportion of motile spermatozoa after cold shock treatment. Sperm motility rates were higher with increased culture time ($p=0.0001$) and with decreased dilutions ($p=0.0003$). Results are represented as mean±SE.

Figure 3. Proportions of sperm with normal acrosomes rate after cold shock treatment. The rates of sperm with normal acrosomes increased with culture time ($p<0.0001$). In the 5-h culture group, the rate of spermatozoa with normal acrosomes decreased with increasing dilution ($p<0.0307$). Results are represented as mean±SE.

interaction was observed between culture time and dilution ratio for the proportion of sperm with normal acrosomes.

Discussion

Our analyses of ejaculated sperm from mature microminipigs compared to those of two other pig breeds showed that although some differences were present, the general properties and morphologies of microminipig sperm were similar to those of common domestic pigs. In addition, the sensitivity of microminipig sperm to cold temperatures during storage was similar to that of sperm from common domestic pig breeds.

Our comparison of the sperm properties of microminipigs with those of common pig breeds identified both similarities and differences. The length of the ejaculation time in microminipigs was comparable to that reported for other pig breeds (31, 32). The sperm of microminipigs had a weakly

alkaline pH, similar to common pig breeds (33) and other strains of mini pigs (34). Quality characteristics such as sperm motility, morphological normality, and acrosome normality varied among individuals but were similar to those for other pig breeds (35-38) and other mini pig strains (33). Ejaculate volume and total number of spermatozoa from microminipigs were smaller than those of standard pig breeds. This is likely to be related to the differences in body size between microminipigs and standard breeds; the mean weight of the microminipigs here was 19.7 ± 1.1 kg, which is about one-twentieth of that of standard breeds (39). Per unit weight, ejaculate volume of microminipigs is greater than that of standard breeds (31, 32, 37, 38), although sperm concentration is lower (31, 32, 37, 38); as a result, sperm count per unit body weight was approximately identical in microminipigs to that of standard breeds (31, 32, 37, 40).

In the present study, we made accurate measurements and assessments of the shapes of sperm from microminipigs and

two standard pig breeds using SEM. We found that the sperm of microminipigs were generally slightly larger than those of Landrace and Duroc pigs for overall length and head width. As sperm head lengths did not vary among the breeds, the differences in total length must be due to differences in tail length. Analysis of the various parts of the tail showed that microminipigs sperm were longer than those of the other two breeds in the neck, middle piece, and main part except for the final length; the total of these differences underpinned the inter-breed differences in tail length. Microminipig sperm length was about 106% of the total length of Duroc sperm; these two breeds showed the largest difference in this study. By comparison, bovine sperm, which are similar in shape and size to porcine sperm (41), are about 130% longer than porcine sperm (42). Thus, the difference in overall length between microminipig and Duroc sperm is much smaller than that between bovine and porcine sperm. The difference between microminipig and Duroc pigs can therefore be simply regarded as a difference among pig breeds.

The resistance of the microminipig sperm to cold shock increased with culture time or with decreased dilution during culture. This pattern of response is similar to that in other pig breeds (30). Thus, the sperm of microminipigs, like those of common breeds (30), show increased resistance to cold shock under the influence of sperm itself and sperm plasma by culturing. The cell membrane of pig sperm is unstable due to the low ratio of cholesterol to phospholipids, and undergoes a phase transition that changes from liquid to gel at high temperatures; it is particularly sensitive to low temperatures (cold shock) below 15°C (43, 44). It has also been noted that seminal plasma proteins are associated with sperm motility and morphological normality (45) and with successful low-temperature storage (46). Our cold shock test results show microminipig sperm have the same properties as those of other pig breeds at low temperatures and can therefore be used for research into the effects of low temperatures and freezing on porcine sperm.

Human and pig sperm have many characteristics in common. For example, a reduction in sperm quality is observed in human sperm during the summer (47), and it is believed that this may result in reduced fertility (48). Similarly, pigs also display a deterioration in semen quality and a reduction in productivity in summer (49, 50). The motility of human sperm ceases when they come into contact with rubber products (51, 52); a similar phenomenon has been identified in pigs (53). Microminipigs are designed to be very small and can be bred in laboratory facilities; these characteristics make them valuable for medical research. It is clear from this study that the properties of microminipig semen are similar to those of other pig breeds. Therefore, microminipigs may also be a valuable model system for investigations in human spermatology, including assisted reproductive technologies.

This study provides the first characterization of sperm ejaculated by microminipigs. Due to their small body size, the amount of sperm and the number of sperm produced by microminipigs are reduced compared to larger breeds, but our analyses demonstrate that sperm morphology, motility, and sensitivity to low temperatures are similar to those of standard pigs. This information will be of value for breeding of microminipigs. In addition, microminipigs will undoubtedly prove useful both for medical experiments and also investigations into sperm preservation for application to other pig breeds.

Conflicts of Interest

The Authors declare that no conflicts of interest exist.

Authors' Contributions

Designed the study: TK. Performed research: TK, SE and MO. Collected and analyzed data: TK, SE and MO. Wrote the paper: TK.

References

- 1 Kaneko N, Itoh K, Sugiyama A and Izumi Y: Microminipig, a non-rodent experimental animal optimized for life science research: preface. *J Pharmacol Sci* 115(2): 112-114, 2011. PMID: 21258171. DOI: 10.1254/jphs.10r16fm
- 2 Kawaguchi H, Yamada T, Miura N, Takahashi Y, Yoshikawa T, Izumi H, Kawarasaki T, Miyoshi N and Tanimoto A: Reference values of hematological and biochemical parameters for the world smallest microminipigs. *J Vet Med Sci* 74(7): 933-936, 2012. PMID: 22362255. DOI: 10.1292/jvms.11-0571
- 3 Miura N, Kucho K, Noguchi M, Miyoshi N, Uchiumi T, Kawaguchi H and Tanimoto A: Comparison of the genomic sequence of the microminipig, a novel breed of swine, with the genomic database for conventional pig. *In Vivo* 28(6): 1107-1111, 2014. PMID: 25398807.
- 4 Ando A, Imaeda N, Ohshima S, Miyamoto A, Kaneko N, Takasu M, Shiina T, Kulski JK, Inoko H and Kitagawa H: Characterization of swine leukocyte antigen alleles and haplotypes on a novel miniature pig line, Microminipig. *Anim Genet* 45(6): 791-798, 2014. PMID: 25118109. DOI: 10.1111/age.12199
- 5 Enya S, Kawarasaki T, Otake M, Kangawa A, Uenishi H, Mikawa S, Nishimura T, Kuwahawa Y and Shibata M: Preservation and reproduction of microminipigs by cloning technology. *In Vivo* 30(5): 617-622, 2016. PMID: 27566081.
- 6 Kawaguchi H, Yamada T, Miura N, Noguchi M, Izumi H, Miyoshi N and Tanimoto A: Sex differences of serum lipid profile in novel microminipigs. *In Vivo* 27(5): 617-621, 2013. PMID: 23988896.
- 7 Kawaguchi H, Yamada T, Miura N, Ayaori M, Uto-Kondo H, Ikegawa M, Noguchi M, Wang KY, Izumi H and Tanimoto A: Rapid development of atherosclerosis in the world's smallest Microminipig fed a high-fat/high-cholesterol diet. *J Atheroscler Thromb* 21(3): 186-203, 2014. PMID: 24257467. DOI: 10.5551/jat.21246

- 8 Nakamura N, Hatano E, Iguchi K, Sato M, Kawaguchi H, Ohtsu I, Sakurai T, Aizawa N, Iijima H, Nishiguchi S, Tomono T, Okuda Y, Wada S, Seo S, Taura K, Uemoto S and Ikegawa M: Elevated levels of circulating ITIH4 are associated with hepatocellular carcinoma with nonalcoholic fatty liver disease: from pig model to human study. *BMC Cancer* 19(1): 621, 2019. PMID: 31238892. DOI: 10.1186/s12885-019-5825-8
- 9 Murayama N, Kaneko N, Horiuchi K, Ohyama K, Shimizu M, Ito K and Yamazaki H: Cytochrome P450-dependent drug oxidation activity of liver microsomes from Microminipigs, a possible new animal model for humans in non-clinical studies. *Drug Metab Pharmacokinet* 24(4): 404-408, 2009. PMID: 19745566. DOI: 10.2133/dmpk.24.404
- 10 Mogi M, Toda A, Iwasaki K, Kusumoto S, Takehara H, Shimizu M, Murayama N, Izumi H, Utoh M and Yamazaki H: Simultaneous pharmacokinetics assessment of caffeine, warfarin, omeprazole, metoprolol, and midazolam intravenously or orally administered to Microminipigs. *J Toxicol Sci* 37(6): 1157-1164, 2012. PMID: 23208431. DOI: 10.2131/jts.37.1157
- 11 Yoshikawa T, Takahashi Y, Kawaguchi H, Utsunomiya S, Miura N, Izumi H, Miyoshi N and Tanimoto A: A dermal phototoxicity study following intravenous infusion administration of ciprofloxacin hydrochloride in the novel microminipigs. *Toxicol Pathol* 41(1): 109-113, 2013. PMID: 22767873. DOI: 10.1177/0192623312452489
- 12 Higashi E, Ando A, Iwano S, Murayama N, Yamazaki H and Miyamoto Y: Hepatic microsomal UDP-glucuronosyltransferase (UGT) activities in the microminipig. *Biopharm Drug Dispos* 35(6): 313-320, 2014. PMID: 24752421. DOI: 10.1002/bdd.1898
- 13 Ohno K, Tomizawa A, Jakubowski JA, Mizuno M and Sugidachi A: Characterization of platelet aggregation responses in microminipigs: Comparison with miniature pigs and the influence of dual antiplatelet administration of aspirin plus prasugrel. *Thromb Res* 135(5): 933-938, 2015. PMID: 25776467. DOI: 10.1016/j.thromres.2015.02.014
- 14 Guruge KS, Noguchi M, Yoshioka K, Yamazaki E, Taniyasu S, Yoshioka M, Yamanaka N, Ikezawa M, Tanimura N, Sato M, Yamashita N and Kawaguchi H: Microminipigs as a new experimental animal model for toxicological studies: comparative pharmacokinetics of perfluoroalkyl acids. *J Appl Toxicol* 36(1): 68-75, 2016. PMID: 25877231. DOI: 10.1002/jat.3145
- 15 Hamada T, Watanabe Y, Iida K, Sano N and Amano N: Microminipig: A suitable animal model to estimate oral absorption of sustained-release formulation in humans. *Int J Pharm* 584: 119457, 2020. PMID: 32464228. DOI: 10.1016/j.ijpharm.2020.119457
- 16 Kakimoto T, Otsuka A, Kawaguchi H, Ogata K, Tanimoto A and Kanouchi H: Plasma homocysteine concentrations in novel microminipigs. *In Vivo* 28(4): 579-582, 2014. PMID: 24982225.
- 17 Nakagawa Y, Muneta T, Kondo S, Mizuno M, Takakuda K, Ichinose S, Tabuchi T, Koga H, Tsuji K and Sekiya I: Synovial mesenchymal stem cells promote healing after meniscal repair in microminipigs. *Osteoarthritis Cartilage* 23(6): 1007-1017, 2015. PMID: 25683149. DOI: 10.1016/j.joca.2015.02.008
- 18 Nakagawa Y, Sekiya I, Kondo S, Tabuchi T, Ichinose S, Koga H, Tsuji K and Muneta T: Relationship between MRI T1 rho value and histological findings of intact and radially incised menisci in microminipigs. *J Magn Reson Imaging* 43(2): 434-445, 2016. PMID: 26227552. DOI: 10.1002/jmri.24988
- 19 Ozeki N, Kohno Y, Kushida Y, Watanabe N, Mizuno M, Katano H, Masumoto J, Koga H and Sekiya I: Synovial mesenchymal stem cells promote the meniscus repair in a novel pig meniscus injury model. *J Orthop Res* 39(1): 177-183, 2021. PMID: 32886427. DOI: 10.1002/jor.24846
- 20 Kangawa A, Otake M, Enya S, Yoshida T and Shibata M: Normal developmental and estrous cycle-dependent histological features of the female reproductive organs in microminipigs. *Toxicol Pathol* 45(4): 551-573, 2017. PMID: 28580886. DOI: 10.1177/0192623317710012
- 21 Noguchi M, Miura N, Ando T, Kubota C, Hobo S, Kawaguchi H and Tanimoto A: Profiles of reproductive hormone in the microminipig during the normal estrous cycle. *In Vivo* 29(1): 17-22, 2015. PMID: 25600524.
- 22 Noguchi M, Ikeda T, Kawaguchi H and Tanimoto A: Estrus synchronization in microminipig using estradiol dipropionate and prostaglandin F2 α . *J Reprod Dev* 62(4): 373-378, 2016. PMID: 27151362. DOI: 10.1262/jrd.2015-169
- 23 Noguchi M, Hirata M, Kawaguchi H and Tanimoto A: *Corpus luteum* regression induced by prostaglandin F $_{2\alpha}$ in microminipigs during the normal estrous cycle. *In Vivo* 31(6): 1097-1101, 2017. PMID: 29102931. DOI: 10.21873/in vivo.11175
- 24 Kangawa A, Otake M, Enya S, Yoshida T and Shibata M: Histological development of male reproductive organs in microminipigs. *Toxicol Pathol* 44(8): 1105-1122, 2016. PMID: 27770108. DOI: 10.1177/0192623316673495
- 25 Kangawa A, Otake M, Enya S, Yoshida T, Kangawa Y and Shibata M: Spermatogenesis in the microminipig. *Toxicol Pathol* 44(7): 974-986, 2016. PMID: 27301246. DOI: 10.1177/0192623316654586
- 26 King GJ and Macpherson JW: A comparison of two methods for boar semen collection. *J Anim Sci* 36(3): 563-565, 1973. PMID: 4693863. DOI: 10.2527/jas1973.363563x
- 27 Kowarasaki T, Enya S, Otake M, Shibata M and Mikawa S: Reproductive performance and expression of imprinted genes in somatic cell cloned boars. *Anim Sci J* 88(11): 1801-1810, 2017. PMID: 28568977. DOI: 10.1111/asj.12838
- 28 Pursel VG, Johnson LA and Schulman LL: Acrosome morphology of boar Spermatozoa during *in vitro* aging. *J Anim Sci* 38(1): 113-116, 1974. PMID: 4590933. DOI: 10.2527/jas1974.381113x
- 29 Guidelines for Proper Conduct of Animal Experiments, Tokyo, Science Council of Japan, 2016. Available at: <https://www.scj.go.jp/ja/info/kohyo/pdf/kohyo-20-k16-2e.pdf> [Last accessed on November 21, 2021]
- 30 Pursel VG, Johnson LA and Schulman LL: Effect of dilution, seminal plasma and incubation period on cold shock susceptibility of boar spermatozoa. *J Anim Sci* 37(2): 528-531, 1973. PMID: 4748485. DOI: 10.2527/jas1973.372528x
- 31 Baker RD and Dziuk PJ: Semen composition and ejaculation of the boar after pilocarpine administration. *J Reprod Fertil* 8: 255-256, 1964. PMID: 14209126. DOI: 10.1530/jrf.0.0080255
- 32 Oberlender G, Murgas LDS, Zangeronimo MG, Silva AC and Pereira LJ: Influence of ejaculation time on sperm quality parameters in high performance boars. *J Anim Sci Adv* 2(5): 499-509, 2012.
- 33 Kasuya Y and Kawabe K: Studies on storage of boar semen at 5C: The determination of osmotic pressure and pH in the fractionated sperm-rich fraction of boar semen and nine solutions used as the constituents of diluent. *Jpn J Swine Sci* 13(1): 22-26, 1994.

- 34 Shimatsu Y, Uchida M, Niki R and Imai H: Liquid storage of miniature boar semen. *Exp Anim* 51(2): 143-147, 2002. PMID: 12012722. DOI: 10.1538/expanim.51.143
- 35 Biró K, Barna-Vetró I, Pécsi T, Szabó E, Winkler G, Fink-Gremmels J and Solti L: Evaluation of spermatological parameters in ochratoxin A—challenged boars. *Theriogenology* 60(2): 199-207, 2003. PMID: 12749934. DOI: 10.1016/s0093-691x(02)01375-4
- 36 Waberski D, Schapmann E, Henning H, Riesenbeck A and Brandt H: Sperm chromatin structural integrity in normospermic boars is not related to semen storage and fertility after routine AI. *Theriogenology* 75(2): 337-345, 2011. PMID: 20961609. DOI: 10.1016/j.theriogenology.2010.09.004
- 37 Wiedemann I, Maehlmeier A, Jansen S, Sharifi AR and Knorr C: SNP g.1007A>G within the porcine DNAL4 gene affects sperm motility traits and percentage of midpiece abnormalities. *Reprod Domest Anim* 53(2): 401-413, 2018. PMID: 29235179. DOI: 10.1111/rda.13120
- 38 Krupa E, Wolfová M, Krupová Z and Žáková E: Estimation of economic weights for number of teats and sperm quality traits in pigs. *J Anim Breed Genet* 137(2): 189-199, 2020. PMID: 31515873. DOI: 10.1111/jbg.12437
- 39 Niwa T: Breed. In: *Pig Farming Handbook*. Niwa T(eds.). Tokyo, Yokenndo Ltd., pp 8-41, 1994.
- 40 Flowers WL: Genetic and phenotypic variation in reproductive traits of AI boars. *Theriogenology* 70(8): 1297-1303, 2008. PMID: 18640711. DOI: 10.1016/j.theriogenology.2008.06.016
- 41 Garner DL and Hafez ESD: Spermatozoa and seminal plasma. In: *Reproduction in farm animals* 5th edition. Hafez ESD (eds.). Tokyo, Nishimura Co., Ltd., pp 193-212, 1992.
- 42 Kondracki S, Banaszewska D, Wysokińska A and Chomicz J: Sperm morphology of cattle and domestic pigs. *Reprod Biol* 6 Suppl 2: 99-104, 2006. PMID: 17220948.
- 43 Drobni EZ, Crowe LM, Berger T, Anchordoguy TJ, Overstreet JW and Crowe JH: Cold shock damage is due to lipid phase transitions in cell membranes: a demonstration using sperm as a model. *J Exp Zool* 265(4): 432-437, 1993. PMID: 8463792. DOI: 10.1002/jez.1402650413
- 44 Galantino-Homer HL, Zeng WX, Megee SO, Dallmeyer M, Voelkl D and Dobrinski I: Effects of 2-hydroxypropyl-beta-cyclodextrin and cholesterol on porcine sperm viability and capacitation status following cold shock or incubation. *Mol Reprod Dev* 73(5): 638-650, 2006. PMID: 16450405. DOI: 10.1002/mrd.20437
- 45 De Lazari FL, Sontag ER, Schneider A, Moura AAA, Vasconcelos FR, Nagano CS, Mattos RC, Jobim MIM and Bustamante-Filho IC: Seminal plasma proteins and their relationship with sperm motility and morphology in boars. *Andrologia* 51(4): e13222, 2019. PMID: 30592081. DOI: 10.1111/and.13222
- 46 De Lazari FL, Sontag ER, Schneider A, Araripe Moura AA, Vasconcelos FR, Nagano CS, Dalberto PF, Bizarro CV, Mattos RC, Mascarenhas Jobim MI and Bustamante-Filho IC: Proteomic identification of boar seminal plasma proteins related to sperm resistance to cooling at 17°C. *Theriogenology* 147: 135-145, 2020. PMID: 31780059. DOI: 10.1016/j.theriogenology.2019.11.023
- 47 Ozelci R, Yılmaz S, Dilbaz B, Akpınar F, Akdag Cırık D, Dilbaz S and Ocal A: Seasonal variation of human sperm cells among 4,422 semen samples: A retrospective study in Turkey. *Syst Biol Reprod Med* 62(6): 379-386, 2016. PMID: 27611370. DOI: 10.1080/19396368.2016.1225322
- 48 Levitas E, Lunenfeld E, Weisz N, Friger M and Har-Vardi I: Seasonal variations of human sperm cells among 6455 semen samples: a plausible explanation of a seasonal birth pattern. *Am J Obstet Gynecol* 208(5): 406.e1-406.e6, 2013. PMID: 23395928. DOI: 10.1016/j.ajog.2013.02.010
- 49 Murase T, Imaeda N, Yamada H and Miyazawa K: Seasonal changes in semen characteristics, composition of seminal plasma and frequency of acrosome reaction induced by calcium and calcium ionophore A23187 in Large White boars. *J Reprod Dev* 53(4): 853-865, 2007. PMID: 17519520. DOI: 10.1262/jrd.19026
- 50 Peña ST Jr, Stone F, Gummow B, Parker AJ and Paris DBBP: Tropical summer induces DNA fragmentation in boar spermatozoa: implications for evaluating seasonal infertility. *Reprod Fertil Dev* 31(3): 590-601, 2019. PMID: 30414622. DOI: 10.1071/RD18159
- 51 Jones DM, Kovacs GT, Harrison L, Jennings MG and Baker HW: Immobilization of sperm by condoms and their components. *Clin Reprod Fertil* 4(6): 367-372, 1986. PMID: 2439182.
- 52 Tomlinson MJ, Naeem A, Hopkisson JF and Campbell B: Brief communication: assessment and validation of nonspermicidal condoms as specimen collection sheaths for semen analysis and assisted conception. *Hum Fertil (Camb)* 15(3): 140-143, 2012. PMID: 22873172. DOI: 10.3109/14647273.2012.718100
- 53 Ko JC, Evans LE and Althouse GC: Toxicity effects of latex gloves on boar spermatozoa. *Theriogenology* 31(6): 1159-1164, 1989. PMID: 16726633. DOI: 10.1016/0093-691x(89)90084-8

Received November 24, 2021

Revised December 13, 2021

Accepted December 18, 2021