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Original Article

Development of an optimal protocol to induce capacitation of boar spermatozoa *in vitro*

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ABSTRACT In 1951, Colin Russell Austin and Min Chueh Chang identified "capacitation", a special process involving ejaculated spermatozoa in the female reproductive tract. Capacitation is a phenomenon that occurs *in vivo*, but almost all knowledge of capacitation has been obtained from *in vitro* studies. Therefore, numerous trials have been performed to establish *in vitro* capacitation methods for various studies on reproduction. Although a series of studies have been conducted to develop an optimal protocol for inducing capacitation, most have focused on identifying the appropriate chemical compounds to induce the capacitation of boar spermatozoa *in vitro*. Therefore, the purpose of this study was to identify the optimal incubation time for inducing capacitation *in vitro*. Duroc semen was incubated for various periods (60, 90, and 120 min) to induce capacitation. Sperm function (sperm motility, motion kinematic parameters, and capacitation status) was evaluated. The results showed that total sperm motility, rapid sperm motility, progressive sperm motility, curvilinear velocity, and average path velocity significantly decreased in a time-dependent manner. However, the capacitation status did not show any significant changes. Taken together, these results indicate that an incubation time of more than 60 min suppresses sperm motility and motion kinematic parameters. Therefore, we suggest that 60 min may be the best incubation time to induce capacitation without negative effects on sperm motility and motion kinematics in boar spermatozoa *in vitro*.

Keywords: boar, capacitation, *in vitro,* sperm functions, spermatozoa

INTRODUCTION

Infertility is a prevalent concern that affects humans and animals worldwide. The World Health Organization estimated that 15% of couples worldwide have fertility problems, and male infertility accounts for approximately 50% of all infertility cases in the animal industry (Peddinti et al., 2008; Park et al., 2013). Moreover, infertility can cause huge economic losses and the collapse of the animal industry (Watson, 2000). As it is important to investigate male fertility, *in vitro* studies on male fertility have been conducted in various fields (de Angelis et al., 2017; González-Rodríguez et al., 2018). Furthermore, studies related to fertility have been conducted on various animals (Jamsai and O'Bryan, 2011; Fortes et al., 2013). Especially in pigs, studies have been conducted on reproduction (Zigo et al., 2020). In this study, we used boar spermatozoa to investigate reproduction and male fertility. Be-

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cause they have greater physiological similarities with humans than other domestic animals, pigs can be used as organ donors for xenotransplantation or the production of transgenic animals (Romar et al., 2016; Romar et al., 2019). Thus, pigs are among the best animal models for studying male fertility.

Freshly ejaculated spermatozoa encounter different environments when passing through the female reproductive tract. Physiological changes, known as capacitation, occur as a result of various factors in the female reproductive tract (de Lamirande et al., 1997). In addition, sperm motility is a major factor that determines sperm quality and plays a key role in fertilization. However, sperm motility deteriorates over a long period (Alavi and Cosson, 2005; You et al., 2017). Capacitation causes spermatozoa to change their motility and physiology. During capacitation, sperm motility, kinematic motion change, and the membrane of the sperm head are also destabilized. Consequently, capacitated spermatozoa can exclusively undergo the acrosome reaction, which allows them to penetrate the zona pellucida (Austin, 1952; Baker et al., 2012; Kwon et al., 2014; Bae et al., 2019).

Capacitation is an *in vivo* phenomenon (Austin, 1951; Chang, 1951); however, the current knowledge of capacitation has been obtained from *in vitro* studies. Various trials have been performed to establish *in vitro* capacitation methods for reproductive studies, and developing a more accurate and standard protocol may be important for successfully inducing capacitation (Kidder, 2014; Rubessa et al., 2019). Although many studies have been conducted to develop an optimal protocol to induce the capacitation of boar spermatozoa, most studies have focused on identifying the appropriate chemical compounds needed to induce the capacitation of boar spermatozoa *in vitro* (Byrd, 1981; Banerjee and Chowdhury, 1995). Therefore, a new approach is required to ensure optimal capacitation. Few studies have investigated the correlation between incubation time and capacitation, and in general, the incubation time investigated is under 60 min (Herreros et al., 2005; Dapino et al., 2006). Therefore, the purpose of this study is to evaluate whether there is an effect of incubation time (60, 90, and 120 min) on capacitation induction and sperm functions to elucidate the optimal protocol on incubation time for boar spermatozoa *in vitro*.

MATERIALS AND METHODS

Media and chemicals

The medium was prepared as previously described (Kwon et al., 2015; Bae et al., 2019; Bae et al., 2022). Modified tissue culture medium (mTCM) 199 was used as the basic medium (BM) [3.05 mM D-glucose, 2.92 mM calcium lactate, 0.91 mM sodium pyruvate, 10% fetal bovine serum, and 2.2 g/L sodium bicarbonate (Sigma-Aldrich, St Louis, MO, USA)]. We added 10 μg/mL of heparin to induce sperm capacitation.

Sample preparation

All animal procedures were performed in accordance with the guidelines for the ethical treatment of animals and approved by the Institutional Animal Care and Use Committee of Kyungpook National University. Duroc boars were housed in a facility with controlled temperature (20 ± 5℃) and ventilation in Gyeongsan Swine (Gyeongsan, Korea). Twenty-seven Duroc sperm samples were collected from healthy mature Duroc boars using the gloved-hand technique and then diluted (3×10^9 sperm cells/mL) with a broad extender (Beltsville thawing solution). Each experiment was repeated at least 5 times using randomly mixed 3 semen samples for each replicate. The extended semen was stored at 17℃ and processed within 2 h of collection. Three semen samples were mixed to exclude individual variations. Consequently, the samples were incubated with BM (containing 10 μg/mL heparin) for various times (60, 90, and 120 min) at 37℃ and 5% CO_2 to induce capacitation (Bae et al., 2019; Lee et al., 2022).

Sperm motility and motion kinematics

To evaluate sperm motility and motion kinematics, a computer-assisted sperm analysis (CASA) program (FSA2016, Medical supply, Seoul, Korea) with a CMOS image sensor, a 2048 × 1536 (300 M pixels), 60 frame camera (Medical supply, Seoul, Korea), and a BX43 phase-contrast microscope (Olympus, Tokyo, Japan) with a 10 × objective phase-contrast mode were used. A sample of 10 μL (30-40 × 10^6 cells/mL) was smeared on a preheated (37℃) Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel). At least 250 sperm cells were analyzed in five randomly selected fields for each sample. The obtained images were analyzed to determine total sperm

motility (MOT, %), rapid sperm motility (RPD, %), medium sperm motility (MED, %), slow sperm motility (SLW, %), progressive sperm motility (PRG, %), curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity (LIN, %), straightness (STR, %), beat-cross frequency (BCF, Hz), mean angular displacement (MAD, degree), wobble (WOB, %), dance (DNC, μm^2/sec), dance mean (DNM, μm), and amplitude of lateral head displacement (ALH, μm).

Sperm capacitation status

We utilized the H33258/CTC dual staining method (combined Hoechst 33258/chlortetracycline fluorescence assessment) to assess the capacitation status. The sample was centrifuged at 5,000 × g for 5 min. After removing most of the supernatant, 135 μL of DPBS and 15 μL of H33258 solution (10 μg H33258/mL DPBS) were added to the remaining sample and incubated at room temperature (RT) for 5 min. After 5 min, 250 μL of 2% (w/v) polyvinylpyrrolidone was added to the sample. The sample was then washed by centrifugation at 5,000× g for 5 min. The supernatant liquid was removed from the centrifuged samples, and the pellet was resuspended in 500 μL DPBS and 500 μL CTC solution (20 mM Tris, 130 mM NaCl, and 5 mM cysteine, pH 7.4). After refrigerating the sample for 20 min in the dark, 10 mL of the sample was smeared on a glass slide. For each sample, at least 400 spermatozoa were counted on each slide, and a BX43 microscope (Olympus, Tokyo, Japan) with epifluorescence illumination was used as the excitation (BP 340-380/LP 425)/emission (BP 450-490/LP 515) filter (Olympus, Tokyo, Japan). Finally, samples were evaluated and separated into four groups depending on capacitation status: alive and acrosome-reacted sperm (AR pattern, no fluorescence over the head), live and capacitated sperm (B pattern, green fluorescence in the acrosomal region), alive and non-capacitated sperm (F pattern, green fluorescence over the head), and dead sperm (D pattern, blue fluorescence). Consequently, the ratios of the AR, B, and F patterns were calculated based on live spermatozoa.

Statistical analysis

All data were analyzed by one-way ANOVA using SPSS (ver. 25.0, IBM, Armonk, NY, USA). Tukey's multiple comparison test was used to compare three groups. Each experiment was performed at least five times. Data are presented as the mean ± SEM. Statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

The fact that spermatozoa must remain in the female reproductive tract for a certain period to acquire fertility was first reported in 1951 by Colin Russell Austin and Min Chueh Chang in rats and rabbits, respectively (Austin, 1951; Chang, 1951). Austin coined the name "capacitation" in 1952, and it is now referred to as *in vivo* capacitation (Austin, 1952). Capacitation can also be fulfilled *in vitro* using appropriate chemical compounds, such as BSA, glycoproteins, and ions (Naz and Rajesh, 2004; Bailey, 2010; Jin and Yang, 2017). To penetrate and fertilize an egg, the sperm must undergo capacitation, which causes many physiological changes in the sperm. As a result, sperm motion kinematics are altered, and capacitated sperm undergo an acrosome reaction; only acrosome-reacted sperm can penetrate the egg (Austin, 1952; Baker et al., 2012; Kwon et al., 2014; Bae et al., 2019). It is important to consider inducing capacitation to achieve successful *in vitro* fertilization.

Capacitation is an important process that occurs after ejaculation in the female reproductive tract. Various studies have been conducted to develop an optimal protocol for inducing capacitation in boar spermatozoa. However, while various chemical compounds have been identified to induce the capacitation of boar spermatozoa *in vitro* (Bailey, 2010; Jin and Yang, 2017), few studies have described the correlation between incubation time and capacitation *in vitro* (Herreros et al., 2005; Dapino et al., 2006). However, it is not clear what is the best incubation time to induce capacitation *in vitro* for boar spermatozoa. Thus, we performed the present study to determine the optimal incubation time for capacitation *in vitro*.

Generally, various incubation time is applied to induce capacitation for mouse, bull, human, and ram spermatozoa *in vitro* in studies related to reproduction (Wattimena, 2006; Ryu et al., 2014; Bae et al., 2019; Sáez-Espinosa et al., 2020). Therefore, the present study was designed to find the optimal incubation time for *in vitro* capacitation of boar among various incubation times (60 min, 90 min, or 120 min) described in previous studies by analysis of sperm motion parameters and morphological changes. Sperm motility and morphological change are a major

factor determining the quality of sperm and have a key role in fertilization. Therefore, analysis of sperm motion parameters and capacitation status are basic approaches in various studies related to reproduction based on capacitation (Bae et al., 2020; Bae and Kwon, 2020; Hwang et al., 2021). Additionally, it has been reported that the sperm motility is change based on incubation time (Alavi and Cosson, 2005; You et al., 2017).

In the present study, we used the CASA program to measure a series of sperm motility and motion kinematic parameters. Among the sperm motility and motion kinematics parameters investigated, MOT (%), RPD (%), PRG (%), VCL (μm/s), and VAP (μm/s) were significantly decreased in a time-dependent manner ($p < 0.05$; Table 1 and Fig. 1). MED (%), SLW (%), VSL (μm/s), LIN (%), STR (%), BCF (Hz), MAD (degree), WOB (%), DNC (μm^2/sec), DNM (μm), and ALH (μm) showed no significant changes over time (Table 1). Furthermore, we conducted H33258/CTC dual staining to determine the effects of incubation time on the capacitation status. The results showed that the AR pattern did not change significantly in a time-dependent manner (Fig. 2A). Pattern B showed no significant time-dependent changes (Fig. 2B). In addition, the F-pattern showed no significant changes in a time-dependent manner (Fig. 2C). The results seem to indicate that the incubation time does not affect the capacitation status of boar spermatozoa up to at least 120 min, while the motility and kinematics decrease in an incubation time-dependent manner. Taken together, it is suggested that an increase in incubation time of more than 60 min has a detrimental effect on sperm motility and motion kinematic parameters. Therefore, we suggest that 60 min may be the best incubation time to induce capacitation without negative effects on sperm motility and motion kinematics of boar spermatozoa *in vitro*. Here, we present our understanding of the effect of incubation time on sperm functions, such as sperm motility, motion kinematic parameters, and capacitation status, in a time-dependent manner and provide better evidence for guidelines to induce capacitation. Therefore, we hope that our results clarify the effects of incubation time and can be used as basic data in experiments related to fertility and reproduction.

Table 1. Sperm motility and kinematics following incubation time

	Incubation time (min)		
	60	90	120
MOT (%)	78.8 ± 1.42[a]	72.28 ± 2.23[a,b]	66.91 ± 1.47[b]
RPD (%)	67.37 ± 1.72[a]	60.92 ± 2.6[a,b]	56.68 ± 1.55[b]
MDM (%)	8.3 ± 0.38	8.07 ± 0.42	6.79 ± 0.33
SLW (%)	3.12 ± 0.26	3.12 ± 0.3	3.38 ± 0.33
PRG (%)	75.68 ± 1.41[a]	68.99 ± 2.4[a,b]	63.46 ± 1.28[b]
VCL (μm/s)	83.16 ± 2.72[a]	76.28 ± 5.49[a,b]	67.17 ± 2.06[b]
VSL (μm/s)	41.3 ± 1.75	37.44 ± 1.65	35.69 ± 1.46
VAP (μm/s)	59.51 ± 2.01[a]	53.3 ± 3.25[a,b]	48.52 ± 1.97[b]
LIN (%)	41.38 ± 1.28	37.62 ± 0.64	37.04 ± 1.23
STR (%)	69.41 ± 1.05	70.7 ± 1.59	73.53 ± 0.65
BCF (Hz)	5.69 ± 0.3	4.99 ± 0.26	4.79 ± 0.14
MAD (degree)	58.85 ± 2.89	52.89 ± 2.4	52.27 ± 2.62
WOB (%)	71.74 ± 1.01	70.42 ± 1.3	72.37 ± 1.02
DNC (μm^2/s)	279.75 ± 20.9	243.65 ± 40.56	174.58 ± 10.59
DNM (μm)	8.02 ± 0.44	8.1 ± 0.78	6.9 ± 0.25
ALH (μm)	3.28 ± 0.15	3 ± 0.28	2.53 ± 0.1

Sperm motility and kinematics are presented as mean ± SEM, n = 5. Different superscripts ([a,b]) within the same row indicate significant differences ($p < 0.05$) in the one-way ANOVA. MOT, Total sperm motility (%); RPD, Rapid sperm motility (%); MED, Medium sperm motility (%); SLW, Slow sperm motility (%); PRG, Progressive sperm motility (%); VCL, Curvilinear velocity (μm/s); VSL, Straight-line velocity (μm/s); VAP, Average path velocity (μm/s); LIN, linearity [%, (VSL/VCL) × 100]; STR, Straightness [%, (VSL/VAP) × 100]; BCF, Beat-cross frequency (Hz); MAD, Mean angular displacement (degree); WOB= Wobble [%, (VAP/VCL) × 100]; DNC, Dance [μm^2/sec, (VCL × ALH)]; DNM, Mean dance [μm, (ALH/LIN)]; ALH, Mean amplitude of head lateral displacement (μm).

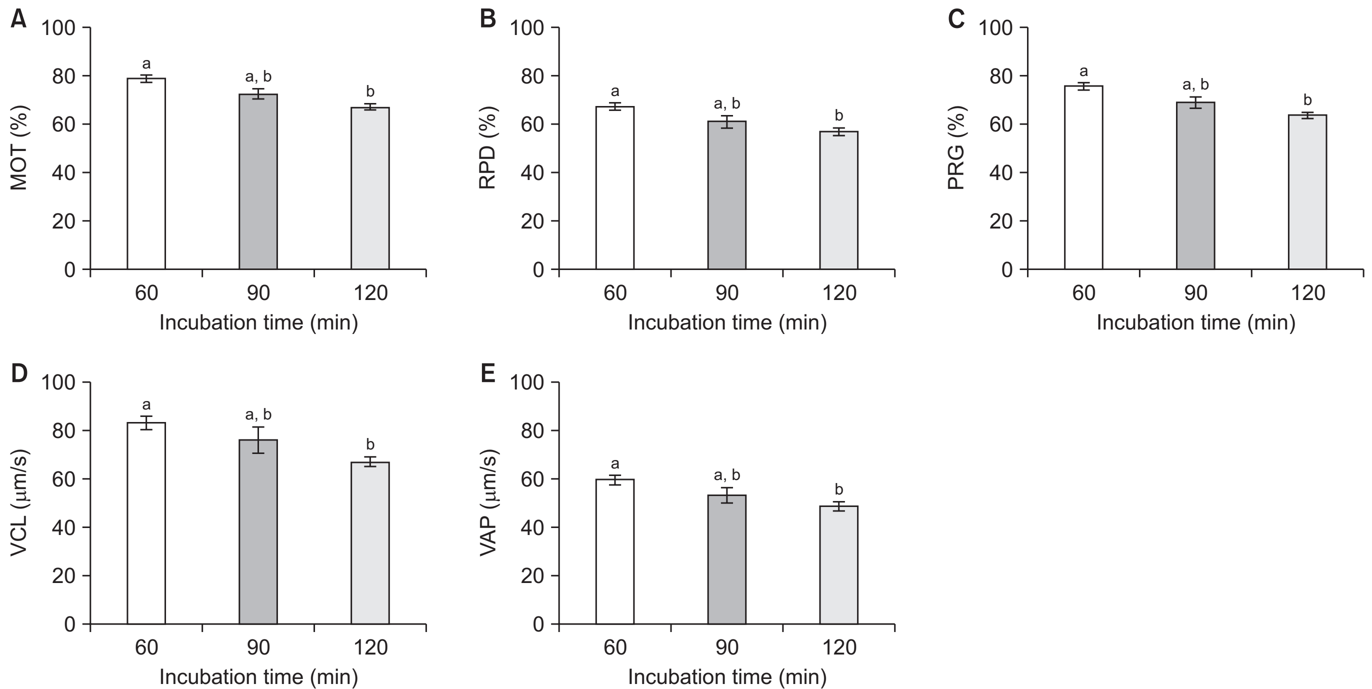

Fig. 1. Sperm motility and kinematics following incubation time. (A) Total Sperm motility. (B) Rapid sperm motility. (C) Progressive sperm motility. (D) Curvilinear velocity (VCL). (E) Average path velocity (VAP). Sperm motility and kinematics are expressed as mean ± SEM, n = 5. Different superscripts (^{a,b}) within the same row indicate significant differences (p < 0.05) determined by one-way ANOVA.

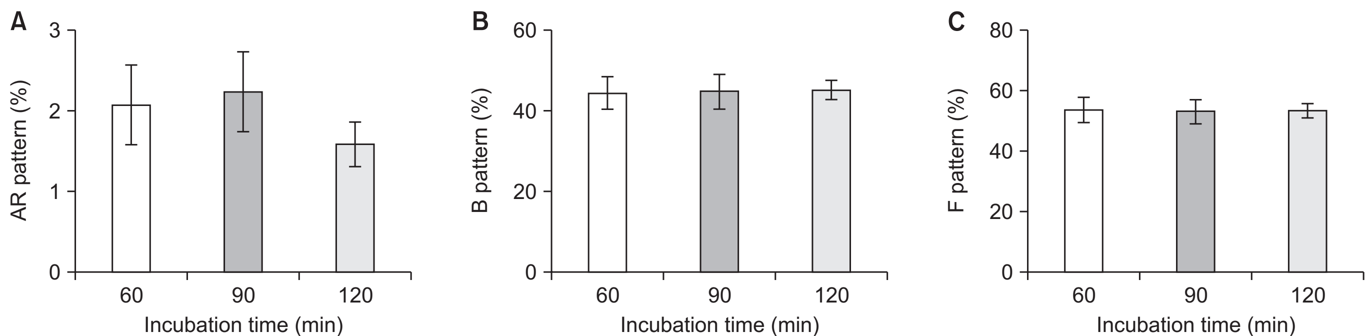

Fig. 2. Sperm capacitation status following incubation time. (A) Patterns of alive and acrosome reacted (AR pattern). (B) Patterns of alive and capacitated (B pattern). (C) Patterns of alive and non-capacitated (F pattern). Values with different superscripts (^{a,b}) indicate significant differences between the control and treatment groups (p < 0.05). Data were evaluated by one-way ANOVA and represent means ± SEM, n = 9.

CONCLUSION

In conclusion, boar spermatozoa MOT (%), RPD (%), PRG (%), VCL (μm/s), and VAP (μm/s) significantly decreased in a time-dependent manner. However, capacitation status showed no significant time-dependent differences. Thus, 60 min may be the best incubation time to induce capacitation and maintain sperm motility and motion kinematics. These findings may be used as basic data for inducing capacitation in various studies related to reproduction.

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REFERENCES

Alavi SM and Cosson J. 2005. Sperm motility in fishes. I. Effects of temperature and pH: a review. Cell Biol. Int. 29:101-110.

Austin CR. 1951. Observations on the penetration of the sperm in the mammalian egg. Aust. J. Sci. Res. B 4:581-596.

Austin CR. 1952. The capacitation of the mammalian sperm. Nature 170:326.

Bae JW, Im H, Hwang JM, Kim SH, Ma L, Kwon HJ, Kim E, Kim MO, Kwon WS. 2020. Vanadium adversely affects sperm motility and capacitation status via protein kinase A activity and tyrosine phosphorylation. Reprod. Toxicol. 96:195-201.

Bae JW, Kim SH, Kim DH, Ha JJ, Yi JK, Hwang S, Ryu BY, Pang MG, Kwon WS. 2019. Ras-related proteins (Rab) are key proteins related to male fertility following a unique activation mechanism. Reprod. Biol. 19:356-362.

Bae JW, Yi JK, Jeong EJ, Lee WJ, Hwang JM, Kim DH, Ha JJ, Kwon WS. 2022. Ras-related proteins (Rab) play significant roles in sperm motility and capacitation status. Reprod. Biol. 22:100617.

Bae JW and Kwon WS. 2020. Investigating the effects of fipronil on male fertility: insight into the mechanism of capacitation. Reprod. Toxicol. 94:1-7.

Bailey JL. 2010. Factors regulating sperm capacitation. Syst. Biol. Reprod. Med. 56:334-348.

Baker MA, Nixon B, Naumovski N, Aitken RJ. 2012. Proteomic insights into the maturation and capacitation of mammalian spermatozoa. Syst. Biol. Reprod. Med. 58:211-217.

Banerjee M and Chowdhury M. 1995. Induction of capacitation in human spermatozoa in vitro by an endometrial sialic acid-binding protein. Hum. Reprod. 10:3147-3153.

Byrd W. 1981. In vitro capacitation and the chemically induced acrosome reaction in bovine spermatozoa. J. Exp. Zool. 215:35-46.

Chang MC. 1951. Fertilizing capacity of spermatozoa deposited into the fallopian tubes. Nature 168:697-698.

Dapino DG, Marini PE, Cabada MO. 2006. Effect of heparin on in vitro capacitation of boar sperm. Biol. Res. 39:631-639.

de Angelis C, Galdiero M, Pivonello C, Garifalos F, Menafra D, Cariati F, Salzano C, Galdiero G, Piscopo M, Vece A, Colao A, Pivonello R. 2017. The role of vitamin D in male fertility: a focus on the testis. Rev. Endocr. Metab. Disord. 18:285-305.

de Lamirande E, Leclerc P, Gagnon C. 1997. Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. Mol. Hum. Reprod. 3:175-194.

Fortes MR, Deatley KL, Lehnert SA, Burns BM, Reverter A, Hawken RJ, Boe-Hansen G, Moore SS, Thomas MG. 2013. Genomic regions associated with fertility traits in male and female cattle: advances from microsatellites to high-density chips and beyond. Anim. Reprod. Sci. 141:1-19.

González-Rodríguez LG, López-Sobaler AM, Perea Sánchez JM, Ortega RM. 2018. Nutrición y fertilidad [Nutrition and fertility]. Nutr. Hosp. 35:7-10.

Herreros MG, Aparicio I, Núñez I, García-Marín L, Gil M, Vega FP. 2005. Boar sperm velocity and motility patterns under capacitating and non-capacitating incubation conditions. Theriogenology 63:795-805.

Hwang JM, Bae JW, Jung EJ, Lee WJ, Kwon WS. 2021. Novaluron has detrimental effects on sperm functions. Int. J. Environ. Res. Public Health 19:61.

Jamsai D and O'Bryan MK. 2011. Mouse models in male fertility research. Asian J. Androl. 13:139-151.

Jin SK and Yang WX. 2017. Factors and pathways involved in capacitation: how are they regulated? Oncotarget 8:3600-3627.

Kidder BL. 2014. In vitro maturation and in vitro fertilization of mouse oocytes and preimplantation embryo culture. Methods Mol. Biol. 1150:191-199.

Kwon WS, Rahman MS, Lee JS, Kim J, Yoon SJ, Park YJ, You YA, Hwang S, Pang MG. 2014. A comprehensive proteomic approach to identifying capacitation related proteins in boar spermatozoa. BMC Genomics 15:897.

Kwon WS, Rahman MS, Ryu DY, Park YJ, Pang MG. 2015. Increased male fertility using fertility-related biomarkers. Sci. Rep. 5:1565.

Lee WJ, Jung EJ, Hwang JM, Bae JW, Kwon WS. 2022. GRP78 plays a key role in sperm function via the PI3K/PDK1/AKT pathway. Reprod. Toxicol. 113:103-109.

Naz RK and Rajesh PB. 2004. Role of tyrosine phosphorylation in sperm capacitation / acrosome reaction. Reprod. Biol. Endocrinol. 2:75.

Park YJ, Kim J, You YA, Pang MG. 2013. Proteomic revolution to improve tools for evaluating male fertility in animals. J. Proteome Res. 12:4738-4747.

Peddinti D, Nanduri B, Kaya A, Feugang JM, Burgess SC, Memili E. 2008. Comprehensive proteomic analysis of bovine spermatozoa of varying fertility rates and identification of biomarkers associated with fertility. BMC Syst. Biol. 2:19.

Romar R, Cánovas S, Matás C, Gadea J, Coy P. 2019. Pig in vitro fertilization: where are we and where do we go? Theriogenology 137:113-121.

Romar R, Funahashi H, Coy P. 2016. In vitro fertilization in pigs: new molecules and protocols to consider in the forthcoming years. Theriogenology 85:125-134.

Rubessa M, Boccia L, Di Francesco S. 2019. In vitro embryo production in buffalo species (Bubalus bubalis). Methods Mol. Biol. 2006:179-190.

Ryu DY, Kim YJ, Lee JS, Rahman MS, Kwon WS, Yoon SJ, Pang MG. 2014. Capacitation and acrosome reaction differences of bovine, mouse and porcine spermatozoa in responsiveness to estrogenic compounds. J. Anim. Sci. Technol. 56:26.

Sáez-Espinosa P, Huerta-Retamal N, Robles-Gómez L, Avilés M, Aizpurua J, Velasco I, Romero A, Gómez-Torres MJ. 2020. Influence of in vitro capacitation time on structural and functional human sperm parameters. Asian J. Androl. 22:447-453.

Watson PF. 2000. The causes of reduced fertility with cryopreserved semen. Anim. Reprod. Sci. 60-61:481-492.

Wattimena J. 2006. Pengaruh waktu inkubasi terhadap pola kapasitasi dan reaksi akrosom spermatozoa domba in vitro [The effect of incubation time on capacitation and acrosome reaction of in vitro ovine spermatozoa]. J. Ilmu Ternak Vet. 11:295-301.

You YA, Kwon WS, Saidur Rahman M, Park YJ, Kim YJ, Pang MG. 2017. Sex chromosome-dependent differential viability of human spermatozoa during prolonged incubation. Hum. Reprod. 32:1183-1191.

Zigo M, Maňásková-Postlerová P, Zuidema D, Kerns K, Jonáková V, Tůmová L, Bubeníčková F, Sutovsky P. 2020. Porcine model for the study of sperm capacitation, fertilization and male fertility. Cell Tissue Res. 380:237-262.