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Effect of Arp2/3 Complex on Sperm Motility and Membrane Structure in Bovine

June-Sub Lee, Yoo-Jin Park, Jin Kim, Md Saidur Rahman, Woo-Sung Kwon, Sung-Jae Yoon, Young-Ah You and Myung-Geol Pang†

Department of Animal Science and Technology, School of Bioresource & Bioscience, Chung-Ang University, Anseong 456-756, Korea

ABSTRACT

Sperm capacitation refers to polymerization of filamentous (F)-actin from globular (G)-actin. While the role of actin-related protein 2/3 (Arp2/3) complex in actin polymerization is well appreciated, the underlying mechanism(s) and its relationship with capacitation are poorly understood. Therefore, to evaluate the potential role of Arp2/3 complex on capacitation, bovine spermatozoa were incubated with multiple doses (1, 10 and 100 μM) of CK-636, an inhibitor of Arp2/3 complex with heparin. The cellular localization of the Arp2/3 complex in spermatozoa was identified by immunohistochemistry, whereas western blot was also applied to detect the protein tyrosine phosphorylation of sperm proteins. Additionally, sperm motility and kinematic parameters were evaluated using a computer-assisted sperm analysis system. CK-636 resulted in significant changes in the ratio of Arp2/3 complex localization between acrosome and equatorial region of the spermatozoa. Short-term exposure of spermatozoa to 100 μM of CK-636 significantly decreased sperm motility, however a non-detectable effect on protein tyrosine phosphorylation was observed during capacitation. On the basis of these results, we propose that Arp2/3 complex is associated with morphological changes during capacitation and compromised sperm motility.

(Key words: Arp2/3 complex, CK-636, Sperm capacitation, Actin polymerization, Bovine spermatozoa)

INTRODUCTION

Mammalian spermatozoa undergo physiological changes to achieve their final fertilizing capacity that was termed as "capacitation" (Chang, 1951; Austin, 1951). Capacitation, as a post-translation modification, is associated with tyrosine phosphorylation (Gadella, 2008; Baker *et al*., 2012) that is related to activation of tyrosine kinase by cylic AMP (Visconti *et al*., 1995; Galantino-Homer and Visconti, 1997), increasing of membrane fluidity (Visconti *et al*., 1995), calcium influx (Handrow *et al*., 1989), and hyper-activated motility. These procedures are able to facilitate the spermatozoa to penetrate the oocyte.

During sperm capacitation, actin polymerization and depolymerization are induced by calcium influx. Sperm capacitation facilitates conversion from G-actin to F-actin in time-dependent manner. Subsequently, F-actin is depolymerized by intracellular calcium influx to induce the acrosome reaction (Brener *et al*., 2003). Especially, actin-related protein 2/3 (Arp2/3) complex has been known as a putative key factor of actin polymerization through the enhancing of actin nucleation that increase the number of actin filaments (Henry *et al*., 1999). The nucleation of Arp2/3 complex is associated with a wide range of cellular processes, such as cell motility (Goley *et al*., 2006), oocytes polarization (Sun *et al*., 2011), early embryo cleavage and blastocyst formation (Sun *et al*., 2013). Although the Arp2/3 complex is present in sperm head and tail, there is little information about the function of Arp2/3 complex in spermatozoa. Therefore, we first investigated the distributional changes of Arp-2/3 complex protein during sperm capacitation in this study. Secondly, we tried to determine the overall role of Arp2/3 complex using CK-636, as an inhibitor of Arp-2/3 complex formation (Nolen *et al*., 2009).

MATERIALS AND METHODS

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† Corresponding author:Phone: +82-31-670-4841, E-mail: mgpang@cau.ac.kr

All procedures were performed according to guidelines for the ethical treatment of animals and approved by Institutional Animal Care and Use Committee in Chung-Ang University.

Preparation of Media

Modified tyrode's albumin lactate pyruvate (mTALP) medium (Parrish *et al.*, 1988), which consisted of 100 mM NaCl, 3.1 mM KCl, 2 mM $CaCal \cdot 2H_2O$, 0.4 mM $MgCl \cdot 6H_2O$, 0.3 mM $Na_2HPO_4 \cdot 12H_2O$, 21.6 mM sodium lactate, 25 mM $NaHCO_3$, 1 mM sodium pyruvate and 0.6% BSA. This medium was added 10 μg/ml heparin to give capacitation condition. Additionally, CK-636 was added in this basic medium (BM) to final molar concentration of 1, 10, and 100 μM.

Semen Source and Sperm Preparation

Frozen semen samples from five individual bulls (Korean native cattle) that represented normal fertility levels were obtained from the Hanwoo Improvement Program of the National Agriculture Cooperative Federation (NACF) of Korea. Bull fertility was calculated by non-return rates (NRRs) according to our previous study; Park *et al.*, 2012. The average NRR of five bulls was 71.21±0.56% (70.19, 73.32, 71.10, 70.33, and 71.13%) (Yoon *et al.*, 2012).

Frozen semen sample were thawed in a 39℃ water bath for 20 sec. Individual thawed semen were divided three groups and pooled to make three representative samples. The samples were centrifuged for 20 min at 400 ×g with a discontinuous Percoll density gradient of 1 ml 90% and 1 ml 45% Percoll to remove extender debris and dead spermatozoa. The top layer suspension was discarded after centrifugation. And then, the sperm pellet was washed with BM (w/o heparin). After washing, the samples were incubated for 20 min at 39 ℃ under 5% CO_2 in air for capacitation in BM that was additionally supplemented with CK-636 (1, 10, and 100 μM). The final sperm concentration was adjusted to 20×10^6 cells/ml. The analysis of each condition was replicated three times.

Immunocytochemistry (ICC)

Sperm sample was placed on a glass slid and allowed to air dry. The spermatozoa were then fixed with 3.7% paraformaldehyde for 30 min at 4℃. After fixation, the cells were washed with Dulbecco's phosphate-buffered saline (DPBS) containing 0.1% Tween 20 (PBS-T) and blocked for 1 hr in blocking solution (5% BSA in PBS-T) at 39℃. The slides were incubated with anti-ARPC2 rabbit polyclonal antibody (Abcam, Cambridge, UK) that diluted with blocking solution 1:1,000 overnight at 4℃. And then slides were washed with DPBS. The slides were incubated for 2 hr at room temperature (RT) with fluorescein isothiocyanate conjugated goat anti-rabbit IgG (Abcam, Cambridge, UK) diluted with blocking solution 1:100. All samples were observed with a Nikon TS-1000 microscope using NIS Elements image software (Nikon).

Computer-Assisted Sperm Analysis (CASA)

The sperm motility (%) was measured using a CASA system (ISAS®v1, Proiser R+D, Valencia, Spain). Briefly 10 μl of sample was placed in a Makler counting chamber (Markler, Israel). The filled chamber was placed on the 39℃ heated stage. Using a 10× objective in phase contrast, the image was relayed, digitized and analyzed by the ISAS® software. The program used for user-defined settings were: frames acquired, 25; frame rate, 20 Hz; minimum particles area (in microns^2), 15; maximum particles areas (in microns^2), 70; progressivity of the straightness percentage (%), 70. The velocity of spermatozoa were characterized on 3 broad categories such as rapid (50 μm/s) > medium (25 μm/s) > slow (10 μm/s).

Western Blot Analysis

Western blotting was performed as described previously (Laemmli, 1970) with some modifications. Aliquots of CK-636 treated with medium (0.6% BSA) spermatozoa were centrifuged at 10,000×g for 5 min and washed twice with DPBS. Sperm pellets were resuspended in Laemmli sample buffer (63 mM Tris, 10% glycerol, 10% sodium dodecyl sulfate, and 5% bromophenol blue) containing 5% 2-mercaptoethanol and incubated for 10 min at RT. After incubation, the soluble and insoluble fractions were separated by centrifugation at 10,000 ×g for 10 min and the supernatants were saved. Samples were subjected to SDS-polyacrylamide gel electrophoresis using a 12% mini-gel system (Amersham, Piscataway, NJ, USA) and the separated proteins were transferred to a polyvinylidene fluoride membrane (Amersham, Piscataway, NJ, USA). The membrane was blocked with 3% blocking agent (Amersham, Piscataway, NJ, USA) for 1 hr at RT. Protein tyrosine phosphorylation was detected by incubation with anti-phosphotyrosine antibody (4G10; Millipore, Billerica, MA, USA) diluted with blocking solution (1:1,000) overnight at 4℃. Next the membrane incubated with a horse-radish peroxidase (HRP) conjugated goat anti-rabbit IgG (Abcam, Cambridge, UK) diluted with blocking solution (1:5,000) for 1 hr at RT. And α-tubulin was detected by incubation with monoclonal anti α-tubulin mouse antibody (Abcam, Cambridge, UK) diluted with blocking solution (1:10,000) for 2 hr at RT. Also, membranes were incubated with a HRP conjugated goat anti-mouse IgG (Abcam, Cambridge, UK) diluted with blocking solution (1:10,000) for 1 hr at RT. Membranes

were washed three times with PBS containing Tween-20. The proteins on the membrane were visualized with an enhanced chemiluminescence (ECL) technique using ECL reagents. All bands were scanned with GS-800 calibrated imaging densitometer (Bio-Rad, Hercules, CA, USA) and analyzed with Quantity One (Bio-Rad, Hercules, CA, USA). Finally, bands were calculated by ratio of tyrosine phosphorylated protein/ α-tublin.

Statistical Analysis

The data were analyzed using One-way ANOVA implemented in SPSS (Version 12.0; Chicago, IL, USA), whereas, a Tukey's test was used to locate differences among replicates. This test compares the responses within different replicates; for a significant difference to be obtained, a consistent and reasonable high magnitude is required between control and treated samples. All the tests were reasonably interpreted with a confidence level of 95% (p<0.05) and the numerical data were represented as mean±SEM.

RESULTS

Change of Arp2/3 Complex Localization Treated with CK-636

Arp2/3 complex localize in the acrosomal region (type 1) or equatorial region (type 2) in the bovine spermatozoa (Fig. 1A). When spermatozoa were treated with CK-636 (1-100 μM), the type 1 pattern was significantly decreased at 100 μM CK-636, while type 2 was significantly increased at 100 μM CK-636 (Fig. 1B).

Effect of CK-636 on Sperm Motility

Inhibition of Arp2/3 complex by CK-636 provide significantly different in percentages of capacitated sperm motility. In particular, sperm motility was significantly decreased at 100 μM CK-636 (p<0.05) (Fig. 2). In the spermatozoa motion parameters, however, ratio of rapid spermatozoa (%) was significantly decreased at 100 μM, while ratio of static spermatozoa (%) was significantly increased at 100 μM CK-636 (p<0.05) (Table 1).

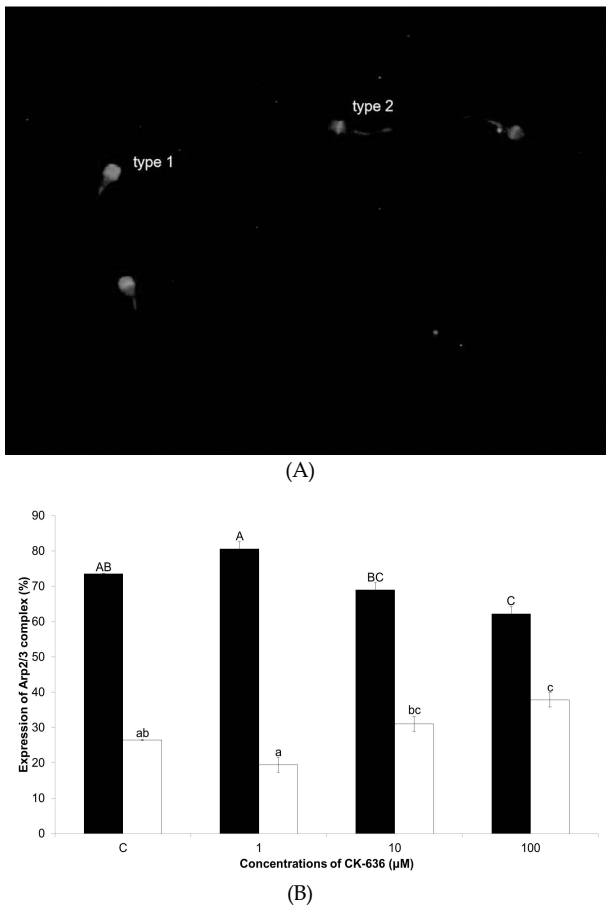

Fig. 1. Localization of Arp2/3 complex in sperm head. (A) Images of ARPC2 (green). There are two type patterns, acrosomal region (type 1) and equatorial region (type 2) in sperm head. (B) Ratio of two type patterns of Arp2/3 complex treated with CK-636, acrosomal region (type1; black bar), and equatorial region (type 2; white bar).

Identification of Tyrosine Phosphorylated Proteins

Because Arp2/3 complex is related to protein tyro-

Table 1. Effect of CK-636 on spermatozoa motion parameters

Parameters (%)	Concentration of CK-636 (μM)			
	C	1	10	100
Motility	66.04±1.90^{a,b}	72.70±3.44^{a}	55.01±4.50^{b,c}	47.20±4.60^{c}
Rapid	57.88±2.01^{a,b}	65.46±3.09^{b}	48.15±2.67^{b,c}	42.54±5.44^{c}
Medium	6.62±2.54	6.33±0.87	4.52±1.33	2.97±0.84
Slow	1.54±0.83	0.90±0.38	2.35±1.08	1.68±0.68
Static	33.93±1.91^{a,b}	27.29±3.44^{b}	45.00±4.50^{b,c}	52.78±4.59^{c}

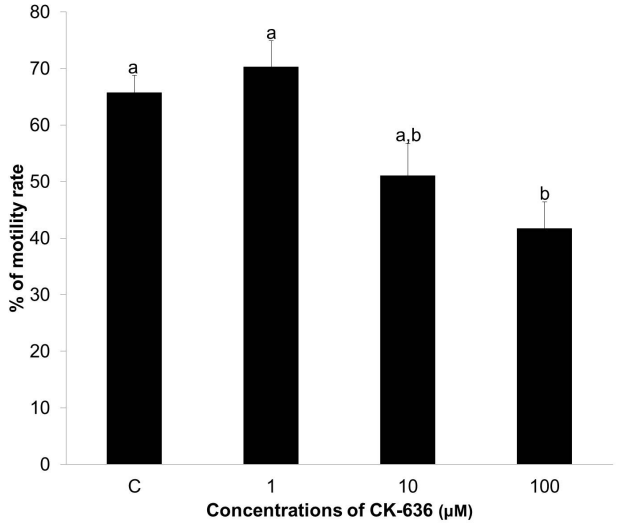

Fig. 2. Effect of CK-636 (inhibitor of Arp2/3 complex) on sperm motility. Changes of motility under CK-636 treatment (1, 10, and100 μM).

sine phosphorylation, we tried to determine the tyrosine phosphorylated protein expression following CK 636 treatment using 4G10 antibody (Gervasi *et al*., 2000). Two different bands (approximately 50 and 16 kDa) were observed in bovine spermatozoa after not only control but also CK-636 treatments (Fig. 3B); however, there were no significantly differences in tyrosine phosphorylated protein expression between control and CK-636 treatment groups ($p<0.05$) (Fig. 3A). Tyrosine phosphorylated proteins were normalized with α-tublin.

DISCUSSION

Actin polymerization has an important role in acquisition of sperm motility during epididymal maturation (Lin *et al*., 2002). Actin polymerization lead to the F-actin formation during sperm capacitation, and then, F-actin is depolymerized by calcium influx. As a consequent result of F-actin depolymerization, sperm outer membrane fusion and the acrosome reaction are induced (Breitbart, 2002; Bernabò *et al*., 2011). Therefore, it is necessary for sperm capacitation, the acrosome reaction, sperm nuclei decondensation, and pronucleus formation in oocyte (Breitbart *et al*., 2005). Arp2/3 complex is one of the actin related proteins that may associate with actin nucleation, polymerization, and depolymerization during sperm capacitation (Brener *et al*., 2003). Interestingly, Arp2/3 complex is abundant in whole sperm head and tail before capacitation while it is observed in post-acrosomal region after capacitation

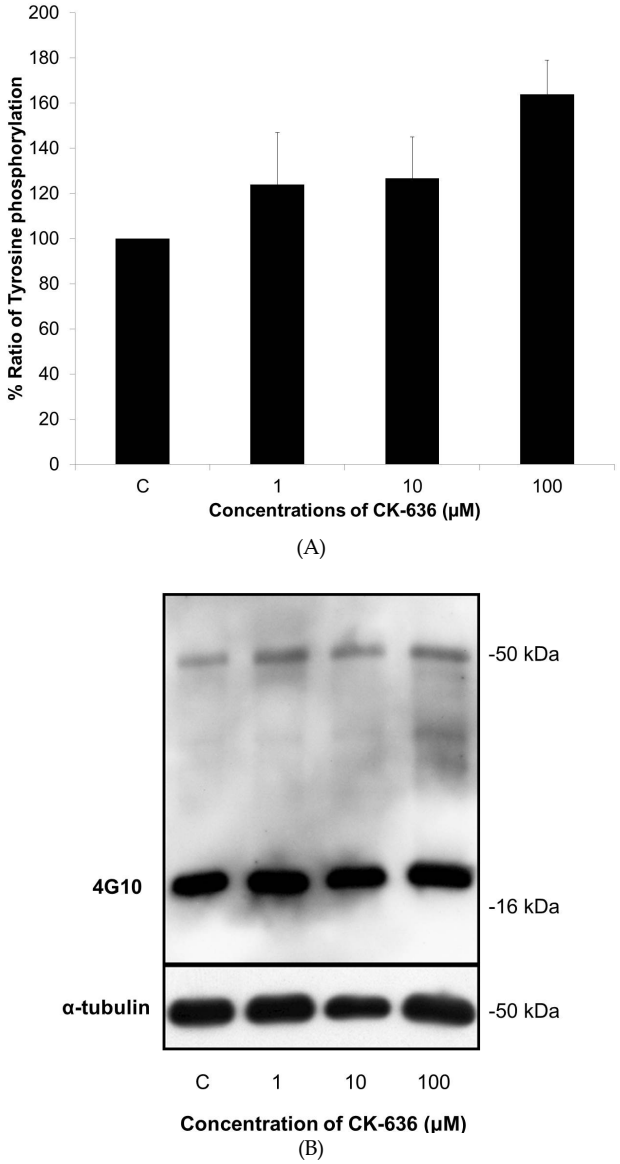

Fig. 3. Effect of CK-636 on tyrosine phosphorylation. (A) Density of tyrosine phosphorylated protein (~16 kDa) in various treatments. (B) tyrosine phosphorylated proteins were probed with 4G-10; lane 1: Control, lane 2: 1 μM CK-636, lane 3: 10 μM CK-636, lane 4: 100 μM CK-636.

(Delgado-Buenrostro *et al*., 2005). However, because there is little information about the role of Arp2/3 complex in spermatozoa, it is difficult to understand the role of Arp2/3 complex in spermatozoa during capacitation. Therefore, this study was to determine the effect of Arp2/3 complex on sperm function during capacitation; first, we identified the localization changes of Arp-2/3 complex protein during sperm capacitation with CK-636 treatment that inhibit of Arp2/3 complex formation in spermatozoa. Secondly, we evaluate the spe-

rm motion parameters and tyrosine phosphorylated proteins after CK-636 treatment during sperm capacitation.

Delgado-Buenrostro *et al.* (2005) reported that Arp2/3 complex were localized in whole the sperm head, while it was identified only in post-acrosomal region following sperm capacitation in guinea pig. However, this study showed that Arp2/3 complex localizes in the acrosomal region (type 1) and equatorial region (type 2) in the bovine spermatozoa (Fig. 1A). Moreover, the type 1 pattern was significantly decreased at 100 μM CK-636, while type 2 was significantly increased at 100 μM CK-636 (Fig. 1B). Based on these findings, we suggested that the differences of Arp2/3 complex distribution in spermatozoa may occur among the different species. Moreover, we hypothesized that the inhibition of Arp2/3 complex may induce the premature acrosome reaction during capacitation.

Sperm motility is a critical parameter for sperm function and male fertility. In this study, sperm motility was significantly decreased at highest concentration compared to control (Fig. 2). Moreover, rapid motility was significantly decreased at highest concentration while static motility was significantly increased (Table 1). These results suggested that Arp2/3 complex may regulate the sperm motility during capacitation through the actin polymerization.

Many investigators have been suggested that actin polymerization is associated with sperm capacitation and tyrosine phosphorylation (Breitbart, 2002), however, in this study, there was no significant difference, tyrosine phosphorylated proteins were increased in CK-636 concentration dependent manner (Fig. 3B). Based on our findings, we suggested that inhibition of Arp2/3 complex may inhibit the sperm motility and induce the premature acrosome reaction through the membrane structural changes, not physiological changes.

In conclusion, we hypothesized that the inhibition of Arp2/3 complex formation is associated with male infertility following the decrease in sperm motility and structural modification of membrane.

REFERENCES

1. Austin CR (1951): Observations on the penetration of the sperm in the mammalian egg. Aust J Sci Res B 4:581-596.
2. Baker MA, Nixon B, Naumovski N, Aitken RJ (2012): Proteomic insights into the maturation and capacitation of mammalian spermatozoa. Syst Biol Reprod Med 58(4): 211-217.
3. Bernabò N, Berardinelli P, Mauro A, Russo V, Lucidi P, Mattioli M, Barboni B (2011): The role of actin in capacitation-related signaling: an *in silico* and *in vitro* study. BMC Syst Biol 5:47.
4. Breitbart H (2002): Intracellular calcium regulation in sperm capacitation and acrosomal reaction. Mol Cell Endocrinol 187:139-144.
5. Breitbart H, Cohen G, Rubinstein S (2005): Role of actin cytoskeleton in mammalian sperm capacitation and the acrosome reaction. Reproduction 129(3):263-268.
6. Brener E, Rubinstein S, Cohen G, Shternall K, Rivlin J, Breitbart H (2003): Remodeling of the actin cytoskeleton during mammalian sperm capacitation and acrosome reaction. Biol Reprod 68(3):837-845.
7. Chang MC (1951): Fertilizing capacity of spermatozoa deposited into the fallopian tubes. Nature 168: 697-698.
8. Delgado-Buenrostro NL, Hernández-González EO, Segura-Nieto M, Mújica A (2005): Actin polymerization in the equatorial and postacrosomal regions of guinea pig spermatozoa during the acrosome reaction is regulated by G proteins. Mol Reprod Dev 70:198-210.
9. Gadella BM (2008): The assembly of a zona pellucida binding protein complex in sperm. Reprod Domest Anim 43:12-19.
10. Galantino-Homer HL, Visconti PE, Kopf GS (1997): Regulation of protein tyrosine phosphorylation during bovine sperm capacitation by a cyclic adenosine 3'5'-monophosphate-dependent pathway. Biol Reprod 56(3):707-719.
11. Gervasi MG, Osycka-Salut C, Caballero J, Vazquez-Levin M, Pereyra E, Billi S, Franchi A, Perez-Martinez S (2011): Anandamide capacitates bull spermatozoa through CB1 and TRPV1 activation. PloS One 6(2): e16993.
12. Goley ED, Welch MD (2006): The ARP2/3 complex: an actin nucleator comes of age. Nat Rev Mol Cell Biol 7(10):713-726.
13. Handrow RR, First NL, Parrish JJ (1989): Calcium requirement and increased association with bovine sperm during capacitation by heparin. J Exp Zool 252(2): 174-182.
14. Laemmli UK (1970): Cleavage of structural proteins during the assembly of the bacteriophage T4. Nature 227:680-685.
15. Lin M, Hess R, Aitken RJ (2002): Induction of sperm maturation *in vitro* in epididymal cell cultures of the tammar wallaby (*Macropus eugenii*): disruption of motility initiation and sperm morphogenesis by inhibition of actin polymerization. Reproduction 124(1):107-117.

16. Nolen BJ, Tomasevic N, Russell A, Pierce DW, Jia Z, McCormick CD, Hartman J, Sakowicz R, Pollard TD (2009): Characterization of two classes of small molecule inhibitors of Arp2/3 complex. Nature 460 (7258): 1031-1034.
17. Park YJ, Mohamed el-SA, Oh SA, Yoon SJ, Kwon WS, Kim HR, Lee MS, Lee K, Pang MG (2012): Sperm penetration assay as an indicator of bull fertility. J Reprod Dev 58(4):461-466.
18. Parrish JJ, Susko-Parrish J, Winer MA, First NL (1988): Capacitation of bovine sperm by heparin. Biol Reprod 38(5):1171-1180.
19. Sun SC, Wang ZB, Xu YN, Lee SE, Cui XS, Kim NH (2011): Arp2/3 complex regulates asymmetric division and cytokinesis in mouse oocytes. PloS One 6(4): e18392.
20. Sun SC, Wang QL, Gao WW, Xu YN, Liu HL, Cui XS, Kim NH (2013): Actin nucleator Arp2/3 complex is essential for mouse preimplantation embryo development. Reprod Fertil Dev 25(4):617-623.
21. Visconti PE, Bailey JL, Moore GD, Pan D, Olds-Clarke P, et al. (1995a): Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. Development 121:1129-1137.
22. Visconti PE, Moore GD, Bailey JL, Leclerc P, Connors SA, et al. (1995b): Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. Development 121:1139-1150.
23. Yoon SJ, Hwang CH, Lee SH, Lee MS, Lee JS, Rahman MS, Kwon WS, Park YJ, You YA, Pang MG (2012): Analysis of estimation of relative conception rate on Korean proven bull number semen using non-return rate. Reprod Dev Biol 36 (3):213-217.

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