Veterinary Integrative Sciences 2022; 20(2): 331-361. DOI; 10.12982/VIS.2022.026

Vet Integr Sci

Veterinary Integrative Sciences

ISSN; 2629-9968 (online)

Website; www.vet.cmu.ac.th/cmvj

Review article

Mammalian sperm capacitation: In vivo and *in vitro* juxtaposition

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Abstract

The development of assisted reproductive technologies (ART) in mammalian species such as *in vitro* embryo production (IVEP) has the potential to provide great benefits for significant population increase, improve genetic performance and advancement, and reduce transmission of venereal diseases. Correspondingly, *in vitro* capacitation of sperm is also paramount, related to the ability of sperm to fertilize oocytes, and was created to imitate in vivo conditions in the female reproductive tract. Amid *in vitro* capacitation developments, studies on how far *in vitro* capacitation has progressed in mimicking in vivo scenes have not been thoroughly reviewed as a comparative form. Therefore, the present study outlined the series of alterations in mammalian sperm capacitation during their journey in the female reproductive tract by exploring and juxtaposing processes under in vivo and *in vitro* conditions. Several essential aspects that become gaps between in vivo and *in vitro* were also identified and elaborated comprehensively in this systematic literature review. We noted that although *in vitro* capacitation procedures in certain mammalian species have made promising progress and improvements, it is still poorly successful in other species like horses. Our findings further postulated that the occurrence of cryocapacitation, the high ratio of capacitated sperm/oocyte required for successful fertilization, and the incidence of polyspermy cause capacitation under *in vitro* settings is less efficient and not yet fully comparable to in vivo. This work is therefore proposed several aspects that need to be bettered from *in vitro* milieu to make it analogous to in vivo environments in modulating sperm capacitation.

Keywords: Capacitation, Cryocapacitation, Mammalia, Polyspermy, Sperm Journey

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Article history; *received manuscript: 27 January 2022, revised manuscript: 20 February 2022, accepted manuscript: 10 March 2022, published online: 18 March 2022*

Academic editor; *Korakot Nganvongpanit*

INTRODUCTION

Assisted reproductive technologies (ART) have been largely involved to support and actualize more efficient and sustainable production of animal-based protein. The current development of ART such as *in vitro* embryo production (IVEP) can significantly increase livestock populations, improve genetic performance and advancement, and reduce transmission of venereal diseases (Ali, 2021). The key point from IVEP is the ability of the sperm to fertilize the oocyte. It is generally accepted that mammalian spermatozoa are unable to fertilize the mature oocyte until they experience successful preparation-related processes prior to fertilization, termed as "capacitation" (Austin, 1952; Chang, 1951). Current information states that only a small percentage of spermatozoa in the ejaculate were reported to retain the potential to achieve fertilization (Oseguera-López et al., 2019).

Capacitation is a complex process which involves several events including cholesterol removal, plasma membrane reorganization, calcium concentration, protein tyrosine phosphorylation, and hyperactivated motility (Kerns et al., 2018; O'Flaherty, 2015). However, how those events are orchestrated is not yet to be clearly understood (Brukman et al., 2019). Another inevitable event is acrosome reaction (AR) which is crucial for sperm to bind and penetrate zona pellucida of matured oocytes (Rathi et al., 2001), also considered as a post-capacitation event (Witte and Schäfer-Somi, 2007). Sperm will undergo capacitation after they are exposed to the new environment in the female reproductive tract which has discrepancies in terms of ion concentration, osmolarity, and pH (Bernecic et al., 2019). To mimic the in vivo condition, some mediums have been developed for *in vitro* capacitation (defined medium) concurrent with the high demand for the commercial production of embryos *in vitro* (Fàbrega et al., 2012; Parrish, 2014).

Several studies claimed that *in vitro* environment is more inefficacious than in vivo since it requires a huge number of sperm for successful fertilization (500 motile sperm/oocyte), reported in humans and mice (Umehara et al., 2018), possibly due to the low sperm quality in which only a small portion of sperm are able to develop the ability to fertilize at any given time under *in vitro* setting (Oseguera-López et al., 2019). However, studies on how far *in vitro* capacitation has been developing in emulating in vivo conditions are not yet fully elucidated and presented as a comparison. Moreover, factors leading to a highly efficient in vivo environment are also necessarily observed to create a similar condition *in vitro* (Umehara et al., 2018). Hence, in this review, we deciphered the sequence changes of the capacitation-related events of mammalian sperm by delving and juxtaposing the processes that occur in the in vivo and *in vitro* conditions as well as highlighting gaps in between.

EARLY DEVELOPMENT OF CAPACITATION IN MAMMALS

The term 'capacitation' firstly emerged from the pioneer study conducted by Austin (1952) through the article entitled "The 'Capacitation' of the Mammalian Sperm" published in Nature Journal on August 23rd, 1952.

However, the initial discovery of the process so-called capacitation has been done independently in rats (Austin, 1951) and rabbits (Chang, 1951) a year before. Austin (1951) reported that the introduction of sperm into the fallopian tube prior to ovulation generated the fertilization of most oocytes while sperm introduced immediately after ovulation lead to the rare penetration of oocytes. These indicated that sperm require some time in the female tract before being capable to penetrate the oocytes (Austin, 1951). Meanwhile, Chang (1951) revealed that fertilization occurs when spermatozoa have undergone physiological changes for 6 hours, observed in doe rabbits following the deposit of sperm suspension in the upper part of tubes through infundibulum with a flank incision. Besides, Bob Edwards exposed human sperm to uterine fluid and analysed its composition by constructing a 2.7 cm diffusion chamber from a nylon tube and inserting it into the uterine. He found that capacitation in human sperm should occur within 12 hours and uterine fluid had more K^+ yet less protein (Edwards et al., 1968). He also stated that the ejaculated cattle sperm were not capable to fertilize oocytes before being incubated with the follicular fluid for 6 – 7 hours, indicating that capacitation is also necessary for cattle's sperm (Bedford, 1970). Ever since then comprehensive experiments on sperm capacitation were massively performed in different mammalian species such as pigs (Hunter and Dziuk, 1968), sheep (Mattner, 1963), golden hamsters (Strauss, 1956), Chinese hamsters (Pickworth and Change, 1969), and primates (Dukelow and Chernoff, 1969) in which incubating sperm in the uterus or oviduct during the oestrus phase became the conventional procedure to achieve capacitation (Kirton and Hafs, 1965).

IN VIVO CAPACITATION

Initial capacitation

Energy Production for Sperm Movement and Its By-product

In the female reproductive tract, mammalian sperm retain linear motility as they move through the uterus to the oviduct using adenosine triphosphate (ATP), generated either via glycolysis (GLY) or oxidative phosphorylation pathway (OXPHOS) (Zhu et al., 2019a). The activation of these metabolic pathways are depending on the metabolic substrates and oxygen availability (Gohil et al., 2010), also very specific among species e.g. glycolysis is crucial in mice, rats, hamsters, and humans spermatozoa for fertilization yet is not so in bovine (Du Plessis et al., 2015) and horse (Leemans et al., 2019). Interestingly, the mitochondrial activity in boar sperm can be induced to generate ATP through OXPHOS in the low glucose condition (Zhu et al., 2019b), switching down the glycolytic pathway (Marroquin et al., 2007) which occurs in the head and flagella of sperm and plays a major role in sperm movement (Figure 1) (Mukai and Okuno, 2004).

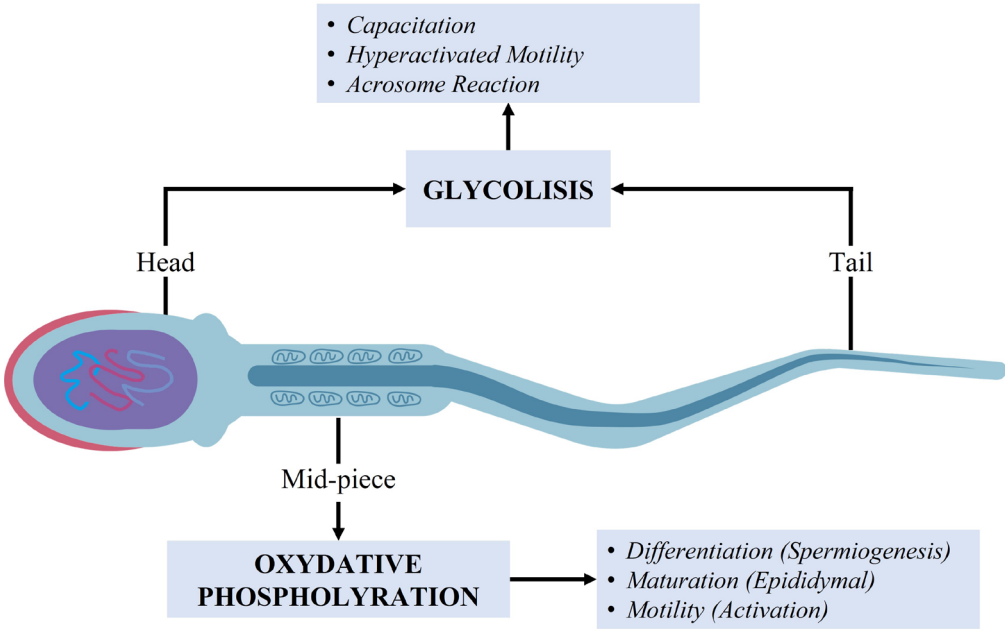

Figure 1 Different metabolic pathways for ATP production, their locations, and the major functions in mammalian sperm.

OXPHOS-based ATP production generates reactive oxygen species (ROS) as a by-product, which acts as a two-edged sword (Aitken, 2017). Low ROS formation is claimed to have an association with sperm capacitation, but higher ROS production can cause oxidative damage by reducing mitochondrial ATP production through enzyme degradation and damaging sperm cell (Aitken and Nixon, 2013; Boerke et al., 2013). In contrast, directing energy generation to glycolysis can reduce oxidative damage and suppress apoptosis (Gohil et al., 2010), maintain ATP levels, sperm kinetic patterns, and oxidative balance in bovine epididymal sperm (Losano et al., 2017). In stallion's sperm, the ATP generation apparently relies on the aerobic pathway of OXPHOS, leading to the high production of ROS and consequently triggering the peroxidation of lipid, DNA damage, and accelerating the sperm death (Gibb et al., 2016; Leemans et al., 2019). However, as a paradox on stallion's fertility, more fertile sperm that resulted in fertilization had a lower percentage of sperm with intact membranes and a greater percentage of sperm with ROS damage compared to sperm that did not result in fertilization (infertile) (Gibb et al., 2014). This contradiction may be attributed to the poor success rate of conventional *in vitro* fertilization (IVF) in horses. Also, this fact has emphasized that sperm responses to the environment that induce capacitation not only differ within the same ejaculate, between males, or before and after cryopreservation, but also among species of mammals (García-Álvarez et al., 2014; Holt and Van Look, 2004). To find out more details regarding the differences of stallion sperm in response to capacitation, we advise the readers to read the review from Leemans et al. (2019).

ROS-induced capacitation in sperm was firstly reported when superoxide anion (O_2^-) prevented motility loss, modulated higher levels of hyperactivation, and capacitation in human (de Lamirande and Gagnon, 1993, 1995) and equine sperm (Baumber et al., 2003). Although the mechanism by

which ROS exerts control over this event has not yet been fully accounted, tyrosine phosphorylation is firmly believed to be the key for controlling sperm capacitation and acrosome reaction, proven by the high increase of tyrosine phosphorylation levels during sperm capacitation in mouse (Aitken, 2017). ROS is necessary to oxidize cholesterol (decapacitation factor), form oxysterol, facilitate efflux at the sperm plasma membrane, and tyrosine phosphorylation events (Aitken, 2011). As sperm were reported to be surrounded by a high concentration of bicarbonate (HCO_3^-) in the female tract (35 mM – 90 mM) (Chaves et al., 2021; Rodriguez-Martinez, 2007), an *in vitro* study in boar and mouse sperm revealed that incubating sperm in bicarbonate-enriched medium induced 10-fold oxysterol formation from cholesterol of the sperm phospholipid membrane via ROS signaling-dependent oxidation of cholesterol (Boerke et al., 2013). Moreover, as oxysterol is more hydrophilic than cholesterol, they can move freely out of sperm membrane through their binding to the proteinaceous acceptor such as albumin, high-density lipoproteins, and apoprotein in the extracellular space (Aitken and Nixon, 2013). In addition, albumin has also been identified as the major protein in the uterus and oviduct fluid secretion, produced from its epithelial which mediates the removal of cholesterol in vivo (Kumaresan et al., 2019).

Oxysterol Removal from Plasma Membrane, Protein Tyrosine Phosphorylation, and Microdomain Aggregation

The cholesterol efflux triggered by ROS leads to the instability of the sperm plasma membrane (Aitken and Nixon, 2013). This condition facilitates the elevation of Ca^{2+} levels intracellular $[Ca^{2+}]_i$ which further stimulates and influences the activity of adenylyl cyclase (SACY/sAC/Adcy10) followed by the activation of the cyclic adenosine monophosphate (cAMP) and the increase of protein kinase A (PKA). Protein tyrosine kinase (PTK) is stimulated yet protein tyrosine phosphatase (Ptyr-Ptase) is inhibited (Fig. 2) (Battistone et al., 2013; Brukman et al., 2019; Parrish, 2014). Consequently, the level of protein tyrosine phosphorylation is elevated, observed in the spermatozoa of humans, rats, mice, bulls, horses, and boars (Aitken, 2017; Boerke et al., 2013).

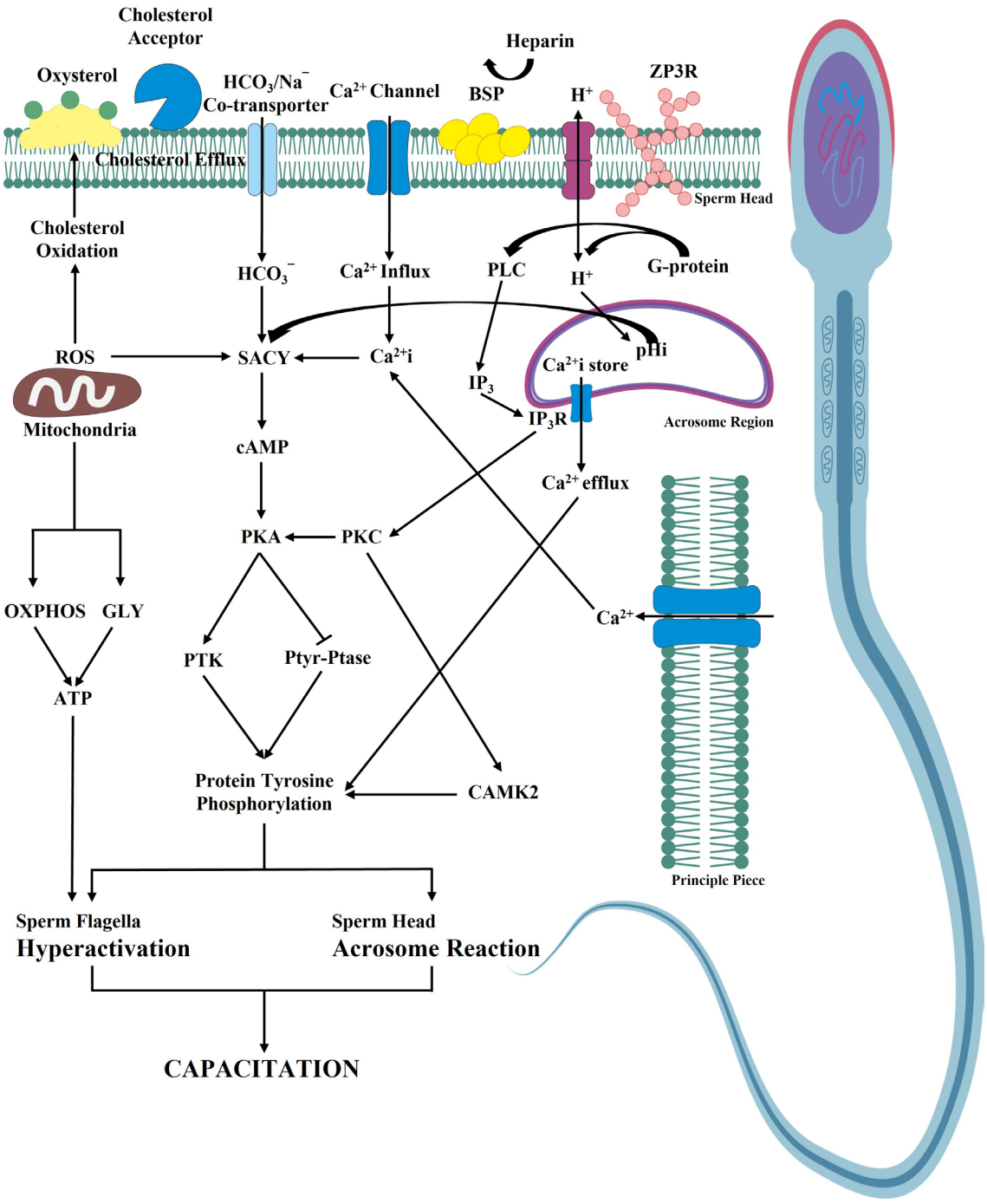

Figure 2 Schematic Illustration of the Capacitation Pathways of Mammalian Sperm.

On the other hand, the presence of bicarbonate and albumin in the female reproductive tract has a synergistic effect to modulate the aggregation of membrane microdomains associated with sperm binding to the zona pellucida (Boerke et al., 2013). From *in vitro* study, it was revealed that incubation of sperm into capacitation medium containing bicarbonate and bovine serum albumin (BSA) modulated cholesterol depletion in the non-raft membrane concomitantly with lipid rearrangement at the raft membrane. At the same time, there was a centralization of complex proteins associated with zona binding (flotillin, caveolin, AQN-3/sperm adhesin, fertilin β, peroxiredoxin 5, etc) (Leemans et al., 2019; van Gestel et al., 2005) and the family of soluble

N-ethylmalmeide-sensitive factor attachment protein receptor (SNARE) proteins involved in the acrosome docking and sperm fusion (VAMP and syntaxin) (Ramalho-Santos et al., 2000) from their initial position distributed in the head of sperm to the apical ridge – a region of sperm head that is undissolved by detergent called as detergent-resistant membrane (DRM) (Gadella and Luna, 2014).

Hypothetically, when sperm are exposed to a bicarbonate-rich environment in the female reproductive tract, the bicarbonate/chloride (HCO_3^-/Cl^-) exchanger is active, and its influx stimulates SACY to raise cAMP levels and activates PKA (Chaves et al., 2021), resulting in phospholipid scrambling which will further lead to cholesterol removal and microdomain aggregation. These processes are called reverse cholesterol transport (RCT). Interestingly, this would not occur if the sperm were in a bicarbonate-deficient environment (epididymis) with an inactive HCO_3^-/Cl^- exchanger (Flesch et al., 2001). Albumin only had a role in depleting cholesterol from the non-raft membrane as the cholesterol level in the raft membrane remained steady following incubation in bicarbonate-enriched medium containing BSA (Leahy and Gadella, 2015; van Gestel et al., 2005). However, a recent study has also shown that cholesterol efflux can be carried out through cholesterol oxidation via ROS-dependent pathway induced by bicarbonate (Boerke et al., 2013). Further theory explained that cholesterol-carrying albumin and its derivative products from sperm membrane will remain in the oviduct before undergoing endocytosis to epithelial cells, evidenced by the discovery of these compounds in the supernatant of *in vitro* capacitation medium after centrifugation (Boerke et al., 2013; Leahy and Gadella, 2015).

The Increase of Calcium Intracellular Uptakes

The intracellular calcium $[Ca^{2+}]_i$ uptake mediates capacitation of mammalian sperm through its function as the second messenger in the most types of cells. Although the transport mechanism of $[Ca^{2+}]_i$ is not well defined, it is hypothesized to be occurred through three possible pathways i.e. CatSper (Cation Channel of Sperm), Ca^{2+} ATPase (Ca^{2+}/H^+ exchanger), or Na^+/Ca^{2+} exchanger in the plasma membrane. Ca^{2+} was unable to enter the sperm in the absence of CatSper (CatSper null sperm), indicating that CatSper channel facilitates Ca^{2+} influx (Navarrete et al., 2015). CatSper is a Ca^{2+}-permeable, pH-dependant, low voltage dependent-channel, and a complex sperm-specific of at least 7 subunits (CatSper 1, 2, 3, 4, β, γ, and δ) localized in principal piece of sperm plasma membrane specifically in the flagella which facilitates subsequent capacitation processes including hyperactivity of sperm flagella and acrosome reaction (Suarez, 2008; Sun et al., 2017).

Sperm-Epithelial Binding

Physical and Molecular Interactions

Upon finishing the initial capacitation in uterine cavity, mammalian sperm head to the utero-tubal junction (UTJ) of the female reproductive tract and establish the interaction that consists of physical and molecular activities. The response of sperm to swim through the fluid flow of the female tract and the contact to microarchitecture of walls as well as the fluid viscoelasticity are

categorized as physical interactions. Meanwhile, the communication between sperm and the receptor in the epithelial linings of female tract is defined as molecular interactions (Suarez, 2016). The apical surface of the epithelial cells surrounding the oviductal lumen is identified as possessing a type of glycoprotein, namely sperm binding glycoprotein (SBG), seen in pig oviduct. It binds to the peri-acrosomal region of capacitating sperm and exposes Galβ1-3GalNAc which will be recognized by boar sperm adhesin AQN1 (Teijeiro et al., 2008) and lactadherin (Mahé et al., 2021). In bovine, sperm-epithelial binding is mediated by Binder of Sperm (BSP1, BSP3, and BSP5) – a protein family located in the sperm acrosome – which interacts to the receptor in the oviduct, namely annexin (ANXA) family protein i.e. ANXA1, ANXA2, ANXA4, and ANXA5 (Ardon et al., 2016). Also, several proteins such as fibronectin and E-cadherin (identified in cattle), GRP78 and HSP60 (identified in human and cattle), and HSPA8 (identified in cattle and pig) have been detected in epithelial cells and are associated with sperm attachment (Mahé et al., 2021).

Reservoir Formation, Its Functions, and Possible Mechanism

Sperm-epithelial interactions occur in the isthmus region of the oviduct, where the "reservoir" is established (Miller, 2015). Sperm reservoir has several essential functions for mammalian sperm including the extension of the sperm lifespan, selection of the competent sperm, and the gradual release of a finite number of fertile sperm population that preclude polyspermy in Mammalia (Mahé et al., 2021; Teijeiro and Marini, 2012). The formation of two main subpopulations of sperm is created in this region, seen in boar spermatozoa. Sperm bind to the epithelial cells of the oviduct (first subpopulation) is considered as the selected high-quality of sperm with normal acrosome morphology, whereas sperm seen in the oviductal lumen (second subpopulation) show membrane alteration (uncapacitated-intact acrosome), poor viability, and degeneration, reported in bovine and porcine sperm (Coy et al., 2010; Teijeiro et al., 2011). How the epithelial cells prolong the viability of sperm is characterized by the inhibition of both capacitation and motility. The interaction of sperm-epithelial cells is believed to decrease the level of $[Ca^{2+}]_i$ uptake thereby maintaining a “low capacitation” state for a period of time (Teijeiro et al., 2008). This suppression is the manifestation of protective effect from sperm-epithelial binding (Suarez, 2016).

The final stage is the release of highly fertile sperm, caused by the alteration of fluid component in the oviduct, cells transcription, the sperm itself near the time (synchronized) with ovulation (Aitken and Nixon, 2013), the existence of peristaltic contraction in oviduct (Ardon et al., 2016; Chang and Suarez, 2012), proteolysis such as sperm adhesin and BSP (particularly BSP3 and BSP5) reported in cattle and pig (Mahé et al., 2021) when sperm are completely capacitated (Miller, 2015), and hormonal milieu. Progesterone (P4) released from ovulated oocyte was reported to stimulate the release of 48% sperm from oviduct cells by increasing the sperm $[Ca^{2+}]_i$ and developing the hyperactivated motility from its action via CatSper channels (Machado et al., 2019). Another evidence reported from *in vitro* study stated that adding nanomolar doses of P4 to co-culture medium containing monolayer of bovine oviduct epithelial cells (BOEC) and sperm resulted in the release of 32%-47% of bull sperm bound to BOEC (Lamy et al., 2017; Mahé et al., 2021).

Hyperactivation

The Change of Sperm Motility Pattern

Hyperactivation/hypermotility/hyperactivated motility is a part of mammalian sperm capacitation which is crucial for achieving fertilization in vivo (Marquez and Suarez, 2004; Suarez, 2008). Hyperactivation aids the capacitated spermatozoa to detach from the reservoir; only sperm exhibiting hyperactivation are able to detach from the epithelial in the mouse (Mahé et al., 2021; Suarez, 2008, 2016). Mammalian sperm also use hyperactivation to give great propulsion to swim through viscoelastic substances in the oviduct such as mucus secreted by oviductal epithelial and cumulus-oocyte complexes (COCs) (Chang and Suarez, 2010). Moreover, hyperactivation takes a role in the penetration to COCs prior to fertilization (Armon and Eisenbach, 2011). The different swimming pattern showed by hyperactivated spermatozoa is characterized by the high-amplitude and asymmetrical flagellar beating (Chang and Suarez, 2012; Hyakutake et al., 2018) which are different from the regularly activated motility of sperm in the uterus (low-amplitude, symmetrical flagellar beating, and a progressive and linear movement) (Chang and Suarez, 2012; Martin-Hidalgo et al., 2018). The presence of Ca^{2+} on the sperm way to oviduct plays a role in the occurrence of hyperactivated motility in most mammalian sperm (Hyakutake et al., 2018) which comes from two possible sources i.e. intracellular calcium $[Ca^{2+}]_i$ stored in organelles and extracellular (pass through a channel on the sperm plasma membrane) (Suarez, 2008).

CatSper Protein Family Mediates Ca^{2+} influx Required for Hyperactivation

Although not fully elaborated, Ca^{2+} influx through CatSper channels and its adhesion to calmodulin have exhibited the transition signal from symmetrical to asymmetrical pattern of sperm flagella at axoneme (Singh and Rajender, 2015), proven by the presence of higher $[Ca^{2+}]_i$ levels in the flagella of hyperactivated sperm than activated sperm in hamster (Chang and Suarez, 2010). Albumin in the oviductal fluid was reported to activate the CatSper channel which increased $[Ca^{2+}]_i$ (Kumaresan et al., 2019; Visconti et al., 2011). CatSper transcripts were found in greater abundance in human sperm samples with high motility than to those with poor motility (Li et al., 2007). The generated mutant male mice lacking any of four CatSper proteins family (CatSper 1, 2, 3, and 4) resulted in infertility, associated with the reduction or failure for hyperactivation and the loss of motility during capacitation (Jin et al., 2007), probably due to the inhibition of necessary Ca^{2+} delivery (Carlson et al., 2005). CatSper1 and Catsper2 were identified as the key component of flagellar channel, expressed in the principal piece of the mature sperm flagella and associated with hyperactivated motility (Carlson et al., 2005). The mice sperm with CatSper1 and CatSper2-null were unable to exhibit hyperactivation criteria and failed to penetrate the zona pellucida, but able to fertilize the zona pellucida-free oocyte (Suarez, 2008). CatSper3 and CatSper4 were also necessary to maintain the sperm motility since the sperm motility defect in those CatSper were seen identic to those seen in CatSper1 and CatSper2 (Jin et al., 2007).

Sperm are exposed to different pH of secretion (mucus) during their travel in vagina (pH ≈ 5), cervix (pH ≈ 8), and fallopian tube (pH ≈ 7.94), and undergo intercellular alkalinization (Ng et al., 2018; Qi et al., 2007). The range of oviductal fluid pH in some mammalian species such as monkey, rabbit, human, and pig, was identified between 7.1 to 8.1 (Ng et al., 2018). The alkalinization promotes a rapid increase of $[Ca^{2+}]_i$ by modulating CatSper or Ca^{2+} channel which, in turn, will trigger the hyperactivated motility of sperm by exhibiting wide flagellar bending. However, how $[Ca^{2+}]_i$ alters sperm flagellar bend is not clearly elucidated (Marquez and Suarez, 2007; Qi et al., 2007). Although electrophysiological data suggest that CatSper proteins are the only source of Ca^{2+} current in mature sperm flagella, other kinds of Ca^{2+} channels such as transient receptor potential (TRPC-1, -3, -4, -6) channels and cyclic nucleotide-gated Ca^{2+} channels identified in sperm flagella may provide Ca^{2+} for hyperactivation without being absolutely necessary (Suarez, 2008).

Sperm Taxis and Hyperactivation

Particular guidance cues take part in navigating mammalian sperm in the female reproductive tract, associated with hyperactivation i.e. (i) Chemotaxis – where chemoattractants released by the cumulus-oocyte complexes (COCs) attract sperm to swim against the chemical gradient (Armon and Eisenbach, 2011; Chang and Suarez, 2010; Mondal et al., 2017); (ii) Thermotaxis – where sperm swim in response to oviductal temperature gradient (Boryshpolets et al., 2015; Mondal et al., 2017); and (iii) Rheotaxis – where the oviduct-uterus fluid flow induce sperm movement to swim against the flow direction (Nagata et al., 2018; Zhang et al., 2016). However, the latter was investigated as a passive process that has no substantial variation in flagellar beating amplitude and asymmetry, reported in human sperm (Zhang et al., 2016).

Chemotaxis

Sperm hyperactivation is enhanced by chemical gradient which guides sperm to reach oocyte (Chang and Suarez, 2010). One of which is progesterone (the primary active agent in follicular fluid) through its oscillations in $[Ca^{2+}]_i$, which causes the rise of $[Ca^{2+}]_i$ and changes in flagellar bending pattern (Machado et al., 2019; Suarez, 2008). The peri-ovulatory follicle or cumulus cells surrounding the oocyte may be the source of progesterone elevation in the female reproductive tract via a counter-current process (Bernecic et al., 2019; Chang and Suarez, 2010; Machado et al., 2019) that is released during ovulation (Coy et al., 2012). The progesterone gradient is formed within and surrounding the cumulus cells which changes sperm flagellar pattern (hyperactivation) and induces acrosome reaction (Chang and Suarez, 2010). Progesterone-induced hyperactivation is associated with phospholipase-C (PLC) which leads to the production of inositol-1,4,5-triphosphate (IP3), the binding of IP3 to IP3R-gated Ca^{2+}-store, the release of $[Ca^{2+}]_i$, and activation of calmodulin-dependent protein kinase-2 (CAMK2) (Fujinoki, 2013). CAMK2 will then increase tyrosine phosphorylation and regulate spontaneous hyperactivation (Fujinoki, 2013; Suarez, 2008). Furthermore, bicarbonate (HCO_3^-) levels in the oviductal fluid secreted from female reproductive tract, particularly

in the ampullary-isthmic junction (AIJ) (Coy et al., 2012; Rodriguez-Martinez, 2007) has also been elucidated to be a prerequisite for hyperactivation by stimulating SACY to generate cAMP (Carlson et al., 2007). The production of cAMP will activate the protein kinase-A (PKA), rising tyrosine phosphorylation in flagella which is correlated with hyperactivated motility in hamster, monkey, and also human sperm (Bernecic et al., 2019; Suarez, 2008). Interestingly, bull sperm was observed to hyperactivate without showing elevation of tyrosine phosphorylation level, suggesting that the increase of tyrosine phosphorylation to regulate hyperactivation is not yet clear (Marquez and Suarez, 2004). Similar results in which hyperactivation and tyrosine phosphorylation occur independently were also demonstrated by mouse and stallion sperm (Leemans et al., 2019).

Thermotaxis

The small temperature gradient between the isthmus and ampulla at ovulation is the basis for thermotaxis (Miki and Clapham, 2013). Recent research on rabbit and human showed that sperm is sensitive to small temperature variation (as small as 0.005 °C or less) (Martin-Hidalgo et al., 2018). The temperature mean of the caudal isthmus in pig is cooler than the cranial ampulla by 0.7 °C (range 0.2 – 1.6 °C) whereas the temperature of sperm storage site is 34.7 °C and fertilization site is 36.3 °C (Hunter, 2012; Ng et al., 2018). A similar condition in the different temperatures between isthmus and ampulla was also reported in rabbit (0.8 – 1.6), human (2.3), and cow (1.5) (Hunter, 2012). Sperm thermotaxis involves two major components; speed enhancement and amplitude rise of flagella seen in human sperm (Boryshpolets et al., 2015). Moreover, temperature increase causes boar sperm to exhibit temporary hyperactivation which will change to progressive motility after a period of time following the stability of temperature (Martin-Hidalgo et al., 2018).

Sperm thermotactic is associated with the rise of $[Ca^{2+}]_i$ levels released from its store (Martin-Hidalgo et al., 2018), yet the primary source of $[Ca^{2+}]_i$ for hyperactivation is extracellular which passes through CatSper channel (Suarez, 2008; Sun et al., 2017). Reduction of extracellular Ca^{2+} will delay the hyperactivation process but increasing pH in the absence of extracellular Ca^{2+} will lead to the formation of mild hyperactivation (Marquez and Suarez, 2007). Further, there is a dynamic equilibrium between extracellular and intracellular Ca^{2+} levels stored in the entire head and midpiece of sperm (Martin-Hidalgo et al., 2018; Yeste et al., 2015). Extracellular and intracellular Ca^{2+} also divided sperm motility pattern into pro- and anti-hook due to the different pathways, reported from *in vitro* study (Chang and Suarez, 2012). The increase of amplitude bend in the same orientation as the hook of the head refers as pro-hook beating, triggered by Ca^{2+} influx intracellular through CatSper and correlated with the rise of pH intracellular (pHi). Meanwhile, the release of $[Ca^{2+}]_i$ modulates the anti-hook beating of mice sperm – a dominant motility pattern over pro-hook which is characterized by an increase of amplitude bend in the opposite way from the hook of sperm head reported from *in vitro* study (Chang and Suarez, 2011).

Acrosome Reaction

The Removal of Sperm Acrosome Region

Capacitation enables mammalian sperm to reach and penetrates the COCs for fertilization (Ickowicz et al., 2012), facilitated by hyperactivated motility (Chang and Suarez, 2010) and acrosome reaction (AR) – a complex, irreversible, and calcium-dependent reaction which releases the contents of secretory vesicle from the anterior portion of the head (acrosome) during early stage of sperm-oocyte interaction through an exocytotic (Simons and Fauci, 2018) (Figure 3). Exocytosis transforms mammalian sperm into a state competent to fertilization but premature exocytosis prevents sperm from entering cumulus cells (Florman et al., 2008). Despite the fact that AR is required for successful fertilization and has been taken into account as part of capacitation, current views regard this process as a post-capacitation event (Visconti et al., 2011). Unlike sperm capacitation which is considered a slow process that takes several hours to complete, AR occurs very quickly (within one minute). However, both require an influx of $[Ca^{2+}]_i$ (Fraser, 1998; Rahman et al., 2014). Two possible ways to trigger AR in vivo have been proposed i.e. the zona pellucida (ZP) (Ickowicz et al., 2012; Kumaresan et al., 2019) and progesterone (secreted from cumulus cells) (Sagare-Patil et al., 2013).

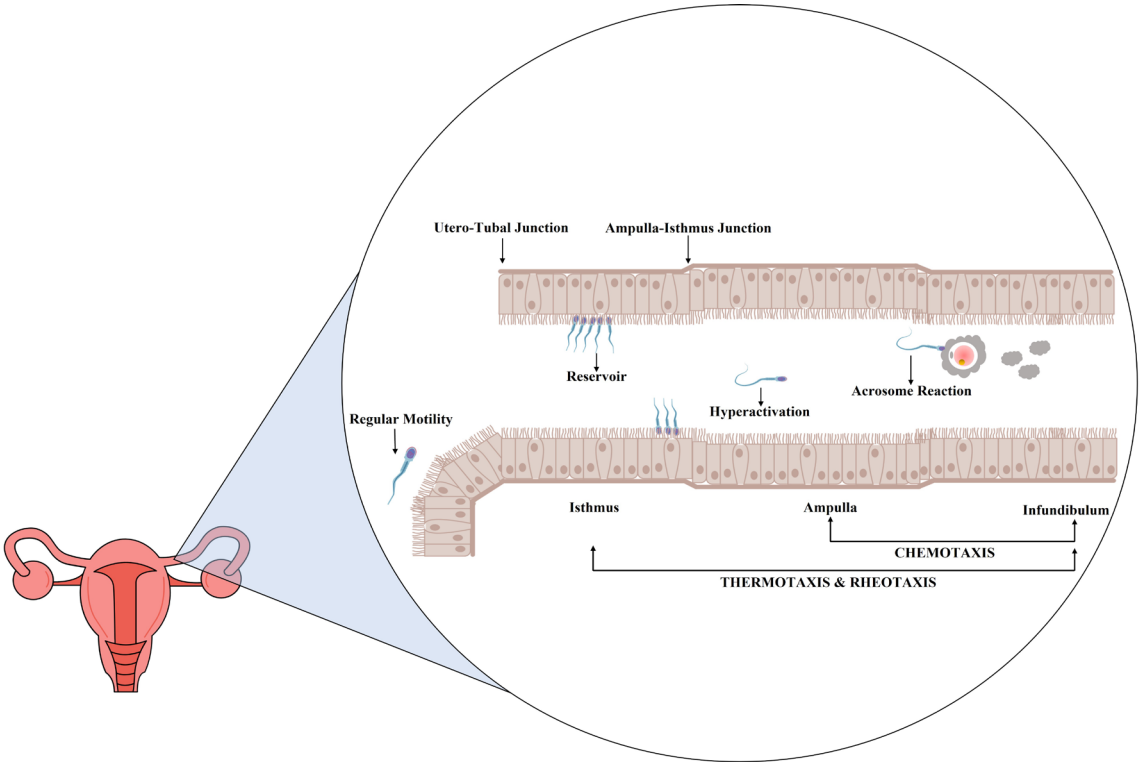

Figure 3 Mammalian Sperm Journey in Female Reproductive Tract During Capacitation.

Zona Pellucida-induced Acrosome Reaction

The anterior head of mice sperm possesses zona pellucida glycoprotein-3 receptor (ZP3R) whereas Gi-coupled receptor and tyrosine kinase receptor are presented in human sperm (Witte and Schäfer-Somi, 2007). In successful fertilization, AR occurs when capacitated sperm interact to the oocyte where zona pellucida glycoproteins are located (Simons and Fauci, 2018). Sperm-ZP binding initiates some necessary responses to facilitate the sustained Ca^{2+} influx, leading to AR. This binding also triggers the activation of G-proteins (Gi1 and Gi2) and elevation of pH intracellular (pHi) in cytosol, leading to a temporary alkalinization (Florman et al., 2008). Although the role of pH in ZP3 action is not really clear, the alkalinization supports Ca^{2+} influx by opening voltage-dependent Ca^{2+} channel which, in turn, increases the Ca^{2+}, modulates protein phosphorylation, and acrosome reaction (Florman et al., 2008; Witte and Schäfer-Somi, 2007). G-protein-coupled also works for enzyme activation of phospholipase-C (PLC) and soluble adenylyl cyclase (SACY) (Boerke et al., 2008; Simons and Fauci, 2018). PLC hydrolyses phosphatidylinositol-4, 5-biphosphate (PIP2) into diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3) (Florman et al., 2008; Simons and Fauci, 2018) while SACY produces cyclic adenosine monophosphate (cAMP) which activates protein kinase A (PKA) for sperm hyperactivation (Bernecic et al., 2019; Boerke et al., 2008; Carlson et al., 2007; Suarez, 2008). Besides, DAG activates the protein kinase-C (PKC) to open calcium channels in plasma membrane, raise the calcium level in cytosol, and trigger AR (Florman et al., 2008). IP3 will bind to its receptor localized in acrosome and mediate the opening of IP3-gated channel, releasing the $[Ca^{2+}]_i$ from acrosome and resulting in further Ca^{2+} depletion as acrosome is reacted (Rahman et al., 2014; Simons and Fauci, 2018).

Progesterone-induced Acrosome Reaction

Recent in vivo studies reported a number of acrosome-reacted sperm in the upper isthmus and 5% sperm with acrosome intact in the ampulla (Hino et al., 2016; Spina et al., 2017), suggesting that the induction of acrosome reaction by ZP is not the main mechanism (Simons and Fauci, 2018). Progesterone (P4) is secreted by cumulus cells surrounding the oocyte which has been widely shown to induce AR (Jin et al., 2011; Simons and Fauci, 2018); also presents in the pre-ovulatory follicular fluid (Witte and Schäfer-Somi, 2007). While pico- or nanomolar P4 concentration modulates the flagellar motility, the higher concentration (micromolar) of P4 promotes AR in sperm (Florman et al., 2008). Although the biological relevance and physiological role of P4 in the modulation of AR are yet unknown, it is associated with protein tyrosine kinase and PKC signaling pathway, and voltage-dependent Ca^{2+} channel (Witte and Schäfer-Somi, 2007). AR induction by P4 does not bypass normal regulatory mechanisms. Instead, it further improves sperm-ZP binding mediating AR induction via specific receptors in the plasma membrane (Neild et al., 2005). A non-genomic P4 receptor (P4R) has been proven by the immunohistochemical studies to be in the acrosome region (Witte and Schäfer-Somi, 2007). Additionally, AR induction pathway via P4 is very species-specific; P4 has no impact on the lipid stability of sperm plasma membrane or the induction of AR but increases capacitation in bovine sperm (Lukoseviciute et al., 2004). Similar observations also failed to give evidence of P4-induced AR in bovine (Thérien and Manjunath, 2003) and human sperm (Sagare-Patil et al., 2013)

IN VITRO CAPACITATION

In vitro Capacitation Medium

Early Development of The Mediums

Sperm capacitation can be done *in vitro* in a defined medium (Aitken and Nixon, 2013; Ded et al., 2010; Macías-García et al., 2015). The first demonstration of *in vitro* capacitation was initially tried by Kirton and Hafs (1965) who developed a medium by aspirating fluid from ligated uteri and found the similarity of reactant(s) in uterine fluid to amylase. Further, β-amylase of phosphate-buffered Locke's solution was used to incubate rabbit sperm for 8 – 12 hours at 38 – 39 °C which resulted in capacitated sperm and 40 embryos (out of 114) were cleavage after being inseminated at 0 – 0.5 hours after ovulation. A different technique was demonstrated by Yanagimachi (1969) who incubated hamster epididymal sperm in Tyrode's medium containing follicular fluid for 3 hours and generated fully capacitated sperm, proven by the loss of acrosome cap, vigorous motility display, and subsequently penetrated zona pellucida of oocyte around 30 – 50 minutes after co-incubation. This new method was then followed by some researchers to develop new medium to capacitate mammalian sperm *in vitro*. One of those was Brackett and Oliphant (1975) who used modified *in vitro* fertilization medium of rabbit to incubate buck sperm for 20 minutes at 38 °C with 5 minutes centrifugation at room temperature (25 °C). This medium modulated capacitation of buck sperm proven by the partial alteration of sperm coating seminal plasma components from immunological assay and the fertilization rate up to 73.9%.

Medium Variation

To date, several common capacitation mediums for mammalian sperm have been developed and modified to generate very well-prepared mammalian sperm and fully mimic the in vivo environment of female reproductive tract. Each of the mediums possesses a different procedure that is adjusted according to the laboratory and the sperm from mammalian species used. Essentially, sperm can be capacitated *in vitro* by incubating in a balanced-salt medium at their physiological temperature with a slightly alkaline pH, containing bicarbonate, calcium, albumin, and energy sources such as glucose, lactate, or pyruvate (Vadnais et al., 2007). While pH is maintained at slightly alkaline with the presence of some buffers and environment modifications including CO_2 and O_2 tension, the osmolarity of *in vitro* capacitation medium is apparently set to be isotonic or slightly hypertonic (Table 1.), which ranges following the distinction of seminal plasma and oviductal fluid (296 – 317 mOsm vs 310 mOsm), seen in rabbit (Brackett and Oliphant, 1975).

Table 1 Chemical Composition of Various *In vitro* Capacitation Medium for Mammalian Sperm

Composition	BO[1]	Sp-TALP[2]	Sp-TALP-H[3]	mTALP[4]	BWW[5]	mW[6]	sOF[7]	HTF[8]	HAM's[9]
NaCl (mM)	112.00	100.00	87.00	100.00	91.06	100.00	107.70	101.60	126.50
KCl (mM)	4.02	3.10	3.10	3.10	4.78	4.70	7.16	4.69	3.83
$CaCl_2$ (mM)	2.25	2.00	2.00	2.00	-	-	1.71	2.04	0.30
NaH_2PO_4 (mM)	0.83	0.30	0.30	0.30	-	-	-	-	-
KH2PO4 (mM)	-	-	-	-	-	-	1.19	0.37	0.61
$NaHCO_3^-$ (mM)	37.00	25.00	10.00	25.00	25.00	25.00	25.07	25.00	20.00
$MgCl_2$ (mM)	0.52	0.40	0.40	0.40	-	1.20	0.49	-	-
$MgSO_4$ (mM)	-	-	-	-	2.44	-	-	0.2	0.63
KPO_4 (mM)	-	-	-	-	1.17	-	-	-	-
KH_2PO_4 (mM)	-	-	-	-	-	-	-	0.37	-
HEPES (mM)	-	10.00	40.00	10.00	21.00	22.00	-	-	-
Glucose (mM)	13.90	-	-	5.00	5.55	5.55	-	2.78	6.11
Sodium pyruvate (mM)	1.25	1.00	1.00	1.00	-	1.00	0.30	0.33	1.00
Sodium lactate (mM)	-	21.60	21.60	21.60	21.55	-	3.30	21.40	-
Calcium lactate (mM)	-	-	-	-	-	-	-	-	0.50
BSA (mg/mL)		6.00	6.00	5.00	7.00	7.00	3.00	7.00	-
Lactic acid hemicalcium salt (mM)	-	-	-	-	1.71	4.80	-	-	-
Kanamycin sulphate (µg/mL)	-	-	-	5.00	-	-	-	-	-
Heparin (µg/mL)	-	10	10	-	-	-	-	-	-
Phenol red (µg/mL)	-	-	-	-	-	-	1.30	-	-
Gentamycin (µg/mL)		50.00	-	50.00	-	-	50.00	-	-
Streptomycin (mg/mL)	-	-	-	-	-	-	-	50	-
Penicillin G (IU/mL)	-	-	-	-	-	-	-	100	-
pH	7.80	7.40	7.40	7.40 – 7.60	*Not mentioned*	7.25 – 8.50	7.20 – 7.30	7.20	8.00
Osmolarity (mOsm/kg)	305	*Not mentioned*	*Not mentioned*	*Not mentioned*	303.17	290 - 300	270 - 280	290 - 300	322.04

Note:

[1]Brackett-Oliphant Medium. This medium was used firstly for rabbit sperm with osmolarity of 305 mOsm/kg and pH 7.8, incubated 15 minutes under 5% CO_2 atmosphere at 38 °C (Brackett and Oliphant, 1975).

[2]Sperm TALP Medium. Suitable for bovine sperm with duration of incubation between 0.25 – 4 hours under 5% CO_2 at 39 °C to maintain pH medium (Parrish et al., 1988, 1989).

[3]Sperm TALP Medium with HEPES. The addition of HEPES allows the incubation process to be done under air atmosphere (Parrish, 2014).

[4]Modified TALP Medium by Suzuki et al., (1994). Initial application in boar sperm, incubated at 37 °C under 5% CO_2 for 90 minutes. The duration and temperature were changed to be only 15 minutes at 39 °C prior to centrifugation and co-culture in the in vitro fertilization medium containing 2 mM caffeine (Suzuki et al., 1999).

[5]Biggers, Whitten, and Whittingham Medium. It is basically an IVF medium for human (Calvo et al., 1993) but can also be used for capacitation of stallion sperm (Pommer et al., 2003). The temperature of incubation was 37 °C under 5% CO_2 for 2 – 3 hours.

[6]Modified Whitten Medium. It is a capacitation medium for murine and stallion sperm (Macías-García et al., 2015)

[7]Synthetic Oviductal Fluid Medium. This medium was developed for embryo culture in vitro (Takahashi and First, 1992), but has been successfully applied as capacitation medium for ram sperm with supplementation of 2% oestrus sheep serum, 1 µL/mL heparin, and 1 µL/mL hypotaurine (García-Álvarez et al., 2014). Sperm capacitation could be seen within 1 hour incubation at 38.5 °C under 5% CO_2 (García-Álvarez et al., 2015).

[8]Human Tubal Fluid Medium. This medium widely used in the manipulation of human and mice gametes. However, the study in stallion sperm revealed that HTF support the capacitation related parameters by inducing high level of tyrosine phosphorylation when incubated in a humidified air atmosphere at 38 °C for 30 and 120 minutes (Arroyo-Salvo et al., 2019).

[9]Ham's F-10 medium. This medium was used to capacitate human spermatozoa with addition of follicular fluid albumin incubated under 5% CO_2 at 37 °C for 2 – 5 hours, specifically to stimulate cholester

Freshly ejaculated semen vs Frozen-thawed semen

One crucial factor that interferes with capacitation is the kind of sperm used. In most mammals, sperm cryopreservation resulted in a substantial proportion of infertile sperm following freezing and thawing due to cryodamage/cryoinjury, leading to impaired transit and low sperm survival in the female reproductive tract. In boar sperm, cryopreservation reduced the fertilization ability to only a half than the fresh sperm (Bailey et al., 2000; Yeste, 2015), eight-times more cryopreserved bovine sperm than fresh sperm was required to achieve successful fertilization in vivo (Shannon and Vishwanath, 1995), and frozen-thawed ram sperm generated 20 – 30% lower fertilization rate compared to the fresh sperm (Maxwell et al., 1993). Those findings indicate that freezing and thawing cause significant alteration in the cell's water volume and substantial mechanical stress on the sperm membrane, particularly over the head and midpiece (Bailey et al., 2000). Besides, sperm ROS facilitates oxysterol production which appears as an early pathway of sperm death (especially when fertilization does not occur) whose mechanism consists of two main pathways: the death of receptor (extrinsic pathway) and mitochondrial (intrinsic pathway) (Kim et al., 2009; Ryan et al., 2005). However, mitochondria apparently appears as the essential mediator which activates intrinsic apoptotic cascade and initiates excessive production of ROS, leading to oxidative DNA damage, motility loss, and cell death (Aitken, 2011; Liu et al., 2009).

Cryopreservation also causes unabated generation of ROS by sperm mitochondria, resulting in the oxidative stress associated with low-temperature storage. Consequently, the spermatozoa's redox balance will shift from peroxynitrite ($ONOO^-$) to superoxide (OH^-) and hydrogen peroxide (H_2O_2) through dismutation, resulting in oxidative stress, reducing the ability for capacitation, acrosome reaction which occurs without the appropriate stimulus (spontaneous AR) (García-Álvarez et al., 2014), or apoptosis (programmed cell death) (Aitken, 2011; Maia et al., 2010). But, spontaneous AR was reported to fertilize ZP-free oocytes without difficulty (Inoue et al., 2003). Cryopreservation also enhances premature capacitation of spermatozoa (cryocapacitation) (Bailey et al., 2000; Watson, 2000) by modifying sperm membranes to be more reactive to their surroundings; frozen-thawed sperm are in a partially capacitated state or show capacitation-like changes (Watson, 1995). Although the mechanism by which this cryocapacitation occurs is not yet fully understood, reorganization of sperm membrane initiated by ROS-induced cholesterol efflux during both capacitation and cryopreservation lead to an influx of $[Ca^{2+}]_i$ which appears to be similar marker. Several studies have further identified some other similarities between capacitation and cryocapacitation (Bailey et al., 2000), as displayed in Table 2.

Table 2 Similar Changes of Spermatozoa during Capacitation and Cryocapacitation

Capacitation	Cryocapacitation
Capacitated (Pattern B) showed by CTC assay	Capacitated (Pattern B) showed by CTC assay
Reorganization and fluidization of sperm plasma membrane	Reorganization and fluidization of sperm plasma membrane
ROS production	ROS production
PTP mediated by cAMP	Showing PTP
Competent for *in vitro* fertilization	Competent for in vitro fertilization

Note: CTC = Chlortetracycline Assay; PTP = Protein Tyrosine Phosphorylation; cAMP = Cyclic Adenosine Monophosphate; ROS = Reactive Oxygen Species

These similarities have brought us to an important decision point: even though both capacitation and cryocapacitation in sperm have the competence to fertilize mature oocytes, the best and most effective option remains to use fresh semen instead of cryopreserved semen as it is related to the level of sperm's ability to fertilize the oocyte as described above, observed in bull, boar, and stallion. Also, a study in buffalo sperm revealed that *in vitro* capacitated sperm had a higher binding ability to zona pellucida than cryocapacitated sperm although the immune localization of tyrosine-phosphorylated protein was similar (Kadirvel et al., 2011). Moreover, cryocapacitation is associated with an increase in intracellular calcium, which has been linked to membrane damage (Pommer et al., 2003), where its sublethal damage results in the protein loss from sperm surface, membrane protein segregation, deactivation of membrane-bound enzymes, and reduced lateral protein transport inside the membrane. Given these similarities, further studies employing proteomics approach are needed to distinguish sperm undergoing capacitation from those experiencing cryocapacitation, particularly to confirm the distribution of some protein complex related to zona pellucida binding that exists in the apical ridge of the sperm head and correlates to the microdomain aggregation (Leahy and Gadella, 2015; Ramalho-Santos et al., 2000; van Gestel et al., 2005).

Sperm Selection Methods

Sperm selection during capacitation in vivo has been tried to be replicated *in vitro* through several ways: (i) Washing technique (Gonçalves et al., 2014; Henkel and Schill, 2003), (ii) Migration-based method including swim-up (Volpes et al., 2016), under-lay, and migration sediment method, (iii) Gradient density-based method combining sperm motility, retention at phase boundaries, and adherence to filter matrices such as percoll (García-Álvarez et al., 2015), PureSperm®, Isolate®, etc (Henkel and Schill, 2003). Apparently, further refinement of the *in vitro* capacitation method in mammals should have also involved the sperm selection stage, where so far it only relies on the utilization of two general methods, namely Percoll density gradient and swim-up. Both methods are solely based on the ability of sperm to move (motility) which does not imply that every motile sperm is of the greatest quality (Said and Land, 2011). Also, some parameters such as apoptosis, DNA integrity, membrane maturation, and sperm ultrastructure must be considered notably due to the presence of centrifugation step in both techniques that generate ROS and has a detrimental effect on sperm quality (Oseguera-López et al., 2019). Based on those notions, several advanced methods and natural approaches to sperm selection have enormous potential use to shift from the old methods, such as magnetic-activated cell sorting (MACS) (Gil et al., 2013; Nadalini et al., 2014), hyaluronic acid (HA) binding, Zeta method, and glass wool filtration (Pessoa et al., 2017). For further insight and better understanding of those advanced techniques above, readers are addressed to read some literature reviewed by (Sharma et al., 2015), (Agarwal et al., 2014), and (Oseguera-López et al., 2019). In addition, natural phenomena linked to molecular interaction between sperm and epithelial cells was also imitated and has excellent potential to be further developed in the improvement of sperm selection methods. It was performed by co-culturing epithelial folds from non-pregnant oviduct of cows with sperm suspension for 30 minutes in a 5% CO_2 incubator at 38.5 °C in TALP medium following replication with TALP containing heparin and demonstrated sperm detachment from the isthmic epithelial following their attachment (Ardon et al., 2016).

Flaws and Improvements

It has been an issue that *in vitro* process for sperm selection and capacitation do not recapitulate in vivo circumstances, is suboptimal, and is less efficient than in vivo environment (Muro et al., 2016; Umehara et al., 2018). Overall, we identified some considerable points which distinguish *in vitro* environment from in vivo i.e. (i) Oviductal fluid contains macromolecular components such as amino acids, carbohydrates, hormones, growth factors, purinergic agents, glycoproteins, neurotransmitters, as well as ions and nutrients that could not be found in most capacitation mediums (Chang and Suarez, 2012; Leese et al., 2008) and may be associated with sperm components in the specific pathway to trigger a better response of sperm capacitation. (ii) The exact composition, variation, dissolved oxygen (DO), pH, ion levels, and temperature have not also been well investigated (Ng et al., 2018). Oxygen tension (pO_2) in the female reproductive tract varies cyclically and minutely, impacted by uterine contractility, hormones, the autonomic nervous system, cardiac pulsatility, and myometrial smooth muscle integrity. The temperature and pH also vary across the tract where the pH rises from the vagina toward the fallopian tubes, indicating heterogeneity in the site-specific microbiome and acid-base buffering at the tissue or cellular level (Ng et al., 2018; Qi et al., 2007; Suarez, 2008). Hormones, the density of pelvic/uterine vascular beds, and the efficacy of heat exchange locally all impact temperature fluctuation which is important for sperm motility and embryo development. For further insight into the dynamic pH, temperature, and oxygen tension in female reproductive tract, readers are suggested to read literature review by Ng et al. (2018). (iii) Oviductal fluid contains mucous secretion which tends to be more viscous than capacitation medium and is presumed to affect the flagellar beating pattern of sperm; capacitated sperm *in vitro* exhibited pro-hook hyperactivation switched their pattern to be anti-hook bend when the viscosity of medium was increased, this condition is identic to the pattern found in vivo; (iv) Sperm-epithelial involves molecular interaction in the form of communication between sperm and the receptor presented in epithelial which could affect sperm flagellar beating and sperm function. Sperm detachment from epithelial maintained normal morphology with high competence of fertilization and its regulation of detached sperm population is also extremely effective in preventing the polyspermy (Coy et al., 2010; Teijeiro et al., 2011; Teijeiro and Marini, 2012).

Although the in vivo condition is considered to be more efficient as one capacitated sperm is enough for the completion of fertilization for each oocyte (Muro et al., 2016), *in vitro* capacitation medium for mammalian sperm has also been continuously refined through the introduction of several capacitation inducers and modification of procedure with different approach, as compiled in Table 3. (García-Álvarez et al., 2015; García-Herreros and Leal, 2014; Lukoseviciute et al., 2004; Parrish et al., 1988). There is also an observable trend where the consideration of the use of capacitation inducers leads to recreating the physiological environment of the female genital tract (Chaves et al., 2021), using substances that have been identified to modulate sperm capacitation in vivo such as oviductal fluid (OF) (Navarro-Serna et al., 2021), creatine (Umehara et al., 2018), and the involvement of hormones (estrogen and progesterone) (García-Álvarez et al., 2015).

Table 3 The Current Substances Introduced to Promote *In vitro* Capacitation in Mammalian Sperm

Substances	Type	Medium	Species	Functions	Sources
Heparin	Additive	Sp-TALP, sOF	Bovine, Ram, Buffalo	Promotes destabilization of sperm plasma membrane, increase calcium uptake intracellular and pH, stimulate sperm hyperactivation, initiates acrosome reaction, stimulate polyspermic fertilization	Parrish (2014) García-Herreros and Leal (2014) García-Álvarez et al. (2015) Gonçalves et al. (2014) Kadirvel et al. (2011)
Penicil-lamine*	Additive	Whitten's, sHTF, mBO, Sp-TALP	Stallion, Bovine, Ram	Stimulate sperm hyperactivation, modulate tyrosine phosphorylation in flagella, increase the percentage of acrosome reaction	Ruiz-Díaz et al. (2020) Kang et al. (2015) El-Shahat et al. (2017)
Hypotaurine*	Additive	Sp-TALP, sOF, mBO	Bovine, Ram	Oxygen Radical Scavanger, Increase sperm motility, penetration rate, and pronuclei formation	García-Álvarez et al. (2015) Kang et al. (2015)
Epinephrine*	Additive	Sp-TALP, mBO	Bovine, Ram	Stimulate sAC, increase cAMP concentration,stimulate sperm motility, induce acrosome reaction, and enhance sperm penetration to oocyte	Gonçalves et al. (2014) Kang et al. (2015)
Caffeine	Additive	Sp-TALP	Ram	Induce Acrosome reaction, stimulate hyperactivation	El-Shahat et al. (2016)
Calcium Ionophore	Additive	mHTF, H-Ham's, Sp-TALP, M2	Human, Ram, Mouse	Induce Acrosome reaction	Moody et al. (2017) Castillo et al. (2019)
Progesterone	Additive	mBWW, HTF, HAM'S, Sp-TALP-H, Sp-TALP	Monkey, Human, Bovine, Ram	Induce acrosome reaction, elevate sperm response to ZP, increase calcium influx, stimulate hyperactivation	Sumigama et al. (2015) Tamburrino et al. (2014) Blengini et al. (2011) Sajeevadathan et al. (2019) Gimeno-Martos et al. (2021)
Estrogen	Additive	M2, TALP	Mouse, Human, Stallion, Ram, Boar	Stimulate cholesterol efflux, promote Ca influx, stimulate tyrosine phosphorylation, increase velocity, and sustains sperm motility, induce acrosome reaction	Sebkova et al. (2012) López-Torres and Chirinos (2017) Gautier et al. (2016) Gimeno-Martos et al. (2021) Ded et al. (2010)
LC	Additive	Sp-TALP	Buck, Bovine	Induce Acrosome Reaction	Parrish et al. (1989) Olivares et al. (2015)
Creatine	Additive	HTF	Mouse	Increase ATP levels, reduce sperm/oocyte ratio for successful fertilization	Umehara et al. (2018)
pOF	Albumin replace-ment	PIG-SUM	Boar	Improve some motion parameters, increase penetration rate	Navarro-Serna et al. (2021)
SNP	Additive	Sp-TALP	Buck	Increase vigorous non-linear motion related to hyperactivation	Olivares et al. (2015)

Note: TALP = Tyrode, Albumin, Lactate, Pyruvate Medium; LC = Lysophophatidylcholine; sHTF = Synthetic Human Tubal Fluid; mHTF = Modified Human Tubal Fluid; H-Ham's = Hepes-buffered Ham's F10; sOF = Synthetic Oviductal Fluid; pOF = Periovulatory Oviductal Fluid; SNP = Sodium Nitroprusside; PIG-SUM = Pig Sperm Swim-Up Medium; mBO = Modified Brackett Oliphant; mBWW = Modified Biggers, Whitten, and Whittingham; M2 = Commercial Fertilizing Medium; * = Usually used in combination solutions named PHE

Essentially, the primary goal of *in vitro* capacitation process is to produce capacitated spermatozoa effectively (Chaves et al., 2021) and efficiently, with intact acrosome membrane, hyperactive motility, and the distribution of protein complexes in the apical ridge of spermatozoa associated with zona-binding and zona-induced acrosome reaction (Gadella and Luna, 2014), so that the direction of spermatozoa movement towards oocyte is not disturbed (proven by high sperm bound to oocyte/ZP) (Boerke et al., 2013). However, the high number of spermatozoa bound to ZP, on the other hand, affects to increase the number of sperm per penetrated oocyte (polyspermy) while also decrease the level of monospermy significantly (Coy et al., 2010), resulting in not only inefficient *in vitro* fertilization (IVF) but also early embryonic death (Coy and Avilés, 2010). The rates of polyspermic fertilization in pig following IVF are up to 75% while 30% and 45% were reported in humans and cattle, respectively (Ballester et al., 2014; Coy and Avilés, 2010; Mahé et al., 2021).

Therefore, by considering a qualified number of capacitated sperm released gradually from the reservoir of the oviduct from in vivo side (Teijeiro and Marini, 2012) and the occurrence of polyspermic fertilization due to the high number of sperm bound to ZP occurred *in vitro*, the efficient *in vitro* capacitation in this study is defined as the lowest number of sperm interacting to zona pellucida resulting to highest fertilization rate so that the odds of polyspermic fertilization can be reduced (Coy and Avilés, 2010). This approach is very useful to be applied especially to males with a low number of motile sperm and has also been attempted to efficiently generate the capacitated sperm for the success of conventional IVF to be equivalent to intracytoplasmic sperm injection (ICSI). *In vitro* studies of mouse sperm revealed a promising result in which a ratio of 20 sperm/3 oocytes co-incubated in microdroplet IVF medium resulted in a fertilization rate of >40% (Hasegawa et al., 2014), whereas another study reported a co-incubation ratio of 5 sperm/oocyte in creatine-enriched IVF medium resulted in a fertilization rate of >60%. Although the percentage of polyspermy was not stated, a blastocyst rate of more than 70% in mice was attained (Umehara et al., 2018). A noticeable result was also revealed from pig, where adding 1% of oviductal fluid from periovulatory phase (pOF) in TALP medium resulted in a reduction of spermatozoa per penetrated oocyte and an increase in monospermic fertilization up to 50%. However, the achieved fertilization rate is just approximately 30% (Zapata-Carmona et al., 2020). In addition, increasing the efficiency of sperm capacitation in relation to the reduction in polyspermic levels during IVF should also involve improving the quality of *in vitro* matured oocytes. This is because polyspermy in mammals not only occurs through an oviduct-based mechanism where the massive arrival of sperm is controlled, it also occurs through an oocyte-based mechanism in which oocytes that are matured *in vitro* fail to undergo zona hardening related to the delay of cortical granule exocytosis to block or maintain single penetration of sperm. Further explanation about polyspermy can be found in the review by Coy and Avilés (2010).

Various Methods to Evaluate *In vitro* Capacitation

Since its discovery nearly a half-century ago, distinguishing capacitated from non-capacitated spermatozoa has remained a problem as a simple yet

easy to validate and interpret method to assess capacitation is not widely used (Ded et al., 2019; Rathi et al., 2001). Several phenomena have been linked to sperm capacitation which apparently can be used as the assessment of capacitation, including ROS generation, cholesterol removal, oxysterol formation, plasma membrane reorganization, cAMP production, calcium concentration, protein tyrosine phosphorylation, and hyperactivated motility (Kerns et al., 2018; O'Flaherty, 2015). Apart from that, acrosome reaction may be used to assess the readiness of sperm to undergo fertilization (Witte and Schäfer-Somi, 2007). Those techniques are presented in Table 4.

Table 4 Techniques Used to Evaluate some events related to Capacitation in Mammalian Sperm

Variable Measured	Techniques	Characteristics	Sources
ROS Generation	Fluorescent probe with CM-H_2DCFDA	The intensity of CM-H2DCFDA fluorescence rises as ROS production increased	Del Olmo et al. (2016) Domínguez-Rebolledo et al. (2010)
	Lucigenin chemiluminescence	Monitors intramitochondrial superoxide anion (O_2^-) generated by the mitochondrial ETC	Liu et al. (2009)
	Luminal-derived chemiluminescence	Detects hydrogen peroxide (H_2O_2) released from mitochondria	Liu et al. (2009)
	Phenol Red Colorimetric Technique	Measures hydrogen peroxide H_2O_2 based on the horseradish peroxidase-dependent oxidation of phenol red	Pick and Keisari (1980) Maia et al. (2010)
	Static Oxidation-Reduction Potential (sORP)	Measures redox imbalance in sperm based on the electrochemical circuit	Hernández-Silva et al. (2020)
Cholesterol Redistribution	Filipin Assay	Based on the specific bond between sperm cholesterol and the fluorescent polyene macrolide	Macías-García et al. (2015)
Cholesterol Efflux	Amplex Red® Enzymatic Assay	Measures the endogenous cholesterol efflux of spermatozoa	Pini et al. (2018)
	Mass Spectrometry	Investigates all lipid species and lipid alteration in the plasma membrane during capacitation	Flesch et al. (2001)
Calcium Influx	Fluorescent Probe Fluo-4 + Flow Cytometry	Identifies sperm category into: - Viable sperm with low $[Ca^{2+}]_i$ - Viable sperm with high $[Ca^{2+}]_i$ - Dead sperm with low $[Ca^{2+}]_i$ - Dead sperm with high $[Ca^{2+}]_i$	Kumaresan et al. (2012)
cAMP Level	Direct cAMP enzyme immunoassay system	Measures the optical density of competitive bond between a polyclonal antibody and cAMP in the sample	Gimeno-Martos et al. (2018)
Protein Tyrosine Phosphorylation	Fluorescein Isothiocyanate Conjugate (FITC) + Flow cytometry	Classifies sperm pattern into: - Pattern E (a short line or triangle of fluoresce in the equatorial segment), - Pattern A (uniform fluorescence over the entire acrosome), and - Pattern EA (fluorescence at both equatorial and anterior acrosomal regions)	Kadirvel et al. (2011) Bernecic et al. (2019)
	Western Blotting	Detects four different molecular weights of tyrosine phosphorylated proteins (21, 38, 85, and 109 kDa)	Kumaresan et al. (2019)

Table 4 Techniques Used to Evaluate some events related to Capacitation in Mammalian Sperm (Cont.)

Variable Measured	Techniques	Characteristics	Sources
Hyperactivation	Computer Assisted Sperm Analysis (CASA)	Defines the occurrence of hyperactivation into: - Boar : ALH>3.5 µm, VCL>97 µm/s, LIN<32%, WOB<71% - Human : ALH≥5.0 µm, VCL≥70 µm/s, LIN≤65% - Cattle : ALH≥5.0 µm, VCL≥150 µm/s, LIN≤50% - Ram : ALH>9.0 µm, VCL>250 µm/s, LIN≤30%	Martin-Hidalgo et al. (2018) Sagare-Patil et al. (2013) Ryu et al. (2019) Bernecic et al. (2019)
Capacitation State	Chlortetracycline (CTC) Fluorescein Isothiocyanate-Conjugate (FITC)	Distinguishes sperm condition into: - Pattern D (Dead Sperm) - Pattern F (Non-capacitated with intact acrosome) - Pattern B (Capacitated with intact acrosome) - Pattern AR (Capacitated with reacted acrosome)	Kwon et al. (2018) Rathi et al. (2001)
Acrosome Reaction	Flow cytometry	Differentiates into several characteristics below: - Live-acrosome-intact sperm (LI): PNA-Alexa 488 (-) and PI (-) - Live-acrosome-reacted sperm (LR): PNA-Alexa 488 (+) and PI (-) - Dead-acrosome-intact sperm (DI): PNA-Alexa 488 (-) and PI (+) - Dead-acrosome-reacted sperm (DR): PNA-Alexa 488 (+) and PI (+)	Thongkham et al. (2021)
	Dip Quick® Spermac®	Divides acrosome condition of mammalian sperm into: - Intact - Reacting - Reacted	Runcan et al. (2014)
	Chlortetracycline (CTC)	Attaches to the hydrophobic region of the sperm surface and detects $[Ca^{2+}]_i$ redistribution in the sperm head during capacitation	Rathi et al. (2001)

Note: ROS = Reactive Oxygen Species; cAMP = Cyclic Adenosine Monophosphate; ALH = Amplitude Lateral Head Displacement; VCL = Velocity Curve Linear; LIN = Linearity; and WOB = Wobble; CM-H_2DCFDA = 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluerescein acetyl ester, ETC = Electron Transport Chain, $[Ca^{2+}]_i$ = Calcium Intracellular, PI = Propidium Iodide, PNA = Peanut Agglutinin

CONCLUSION

Physiologically, sperm do not travel through the female reproductive tract solely in search of oocyte; instead, they prepare themselves in the form of capacitation which consists of sperm communication and selection through the attachment and detachment to/from the epithelial cells of the oviduct as well as sperm transformation (cholesterol removal, plasma membrane reorganization, tyrosine phosphorylation, and microdomain aggregation) directed by certain taxis following ambient temperature and the chemical gradient in the oviduct and cumulus secretion. Those transfigurations drive the occurrence of sperm-oocyte binding, penetration, and fertilization of the mature oocyte facilitated by hyperactivated motility and zona pellucida- or progesterone-induced acrosome reaction. In vivo environment is efficient because one capacitated sperm is sufficient to complete fertilization for each oocyte. As a comparison, *in vitro* capacitation has been refined through the improvement of the incubation circumstances, medium modifications, and supplementation of capacitation

promoters which is optimized according to the laboratory and species-specific from which sperm are collected. These improvements have resulted in nearly efficient results as indicated by the decrease in the ratio of capacitated sperm/oocyte for successful *in vitro* fertilization and the increase of monospermic fertilization in certain mammalian species although it is still far from success in other species like horses. We point out howsoever several aspects that need to be rectified from *in vitro* milieu so as to make it comparable to in vivo circumstances in modulating sperm capacitation. Further study in the *in vitro* capacitation should be focusing on the utilization of advanced sperm selection methods and quality examination that necessarily involves a proteomics approach, identification of components in the female genital tract thoroughly including the composition of secretion fluids, and how to manifest the dynamics of temperature, oxygen tension, pH, and other substances that naturally occur in vivo into *in vitro* protocols. In addition, the approaches to reduce the likelihood of polyspermic fertilization *in vitro* are also paramount to be further developed.

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How to cite this article;

Haris Setiawan, Phongsakorn Chuammitri, Korawan Sringarm, Montira Intanon and Anucha Sathanawongs. Mammalian sperm capacitation: *In vivo* and *in vitro* juxtaposition . Veterinary Integrative Sciences. 2022; 20(2): 331- 361.