Review

Research Progress on the Microregulatory Mechanisms of Fertilization: A Review

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Abstract. *The process of fertilization includes sperm capacitation, hyperactivation, an acrosome reaction and the release of acrosome enzymes, membrane fusion and channel formation, the release of the sperm nucleus, and gamete fusion. This process is closely related to the shape and vitality of the sperm, acrosome enzyme release, and the zona pellucida structure of the egg, as well as the opening and closing of various ion (e.g., calcium) channels, the regulation of signaling pathways such as cyclic adenosine monophosphate-protein kinase A, the release of progesterone, and the coupling of G-proteins. The interaction among multiple factors and their precise regulation give rise to multiple cascading regulatory processes. Problems with any factor will affect the success rate of fertilization. Recent studies have shown that with rapid societal development, the incidence of male infertility is increasing and occurs at younger ages. According to World Health Organization statistics, 15% of couples of childbearing ages have infertility problems, of which 50% are caused by male factors. Additionally, the cause of infertility cannot be identified in as many as 60% to 75% of male infertility patients. In this article, we review the research progress on the microregulation of fertilization and mechanisms underlying this process to identify causes and develop novel prevention and treatment strategies for male infertility.*

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Process of fertilization begins with sperm capacitation. When sperm leave the male reproductive tract, they must undergo a maturation process in the female reproductive tract during which they acquire the ability to fertilize. This process is called sperm capacitation. Capacitation is reversible; if a capacitated sperm comes into contact with seminal plasma and epididymal fluid, it can be decapacitated again. Hyperactivation is a necessary condition for fertilization and a capacitation marker, and elevated pH in the extracellular environment is crucial for sperm capacitation. From the time of capacitation to fertilization, both the sperm's external and intracellular environments undergo a shift from acidic to basic, and sperm adapt to this change through numerous biochemical changes. The epididymis has a low pH, high concentration of K^+, and low concentration of Na^+ and HCO_3^-, and this environment determines the intracellular pH of sperm in the epididymis (<6.0). Mammalian sperm remain in a quiescent state in the acidic environment at the tail of the epididymis and vas deferens. As part of its sperm transfer and storage functions, the epididymis secretes de-energizing factors that attach to the sperm surface, temporarily impairing its fertilization capacity. The pH of the uterine cavity is >7.0, and the pH of the fallopian tube is >8.0. In an alkaline environment, sperm capacitation, hyperactivation, an increase in intracellular Ca^{2+}, protein phosphorylation, and other changes can occur. The capacitation process includes plasma membrane reorganization, changes in protein phosphorylation, and membrane hyperpolarization, as well as elevation of intracellular pH and Ca^{2+}. Several molecules, such as serum proteins, Ca^{2+}, and HCO_3^-, are involved in the process of capacitation. These molecules help enable sperm capacitation *via* the regulation of adenylyl cyclase/cyclic adenosine monophosphate (cAMP),
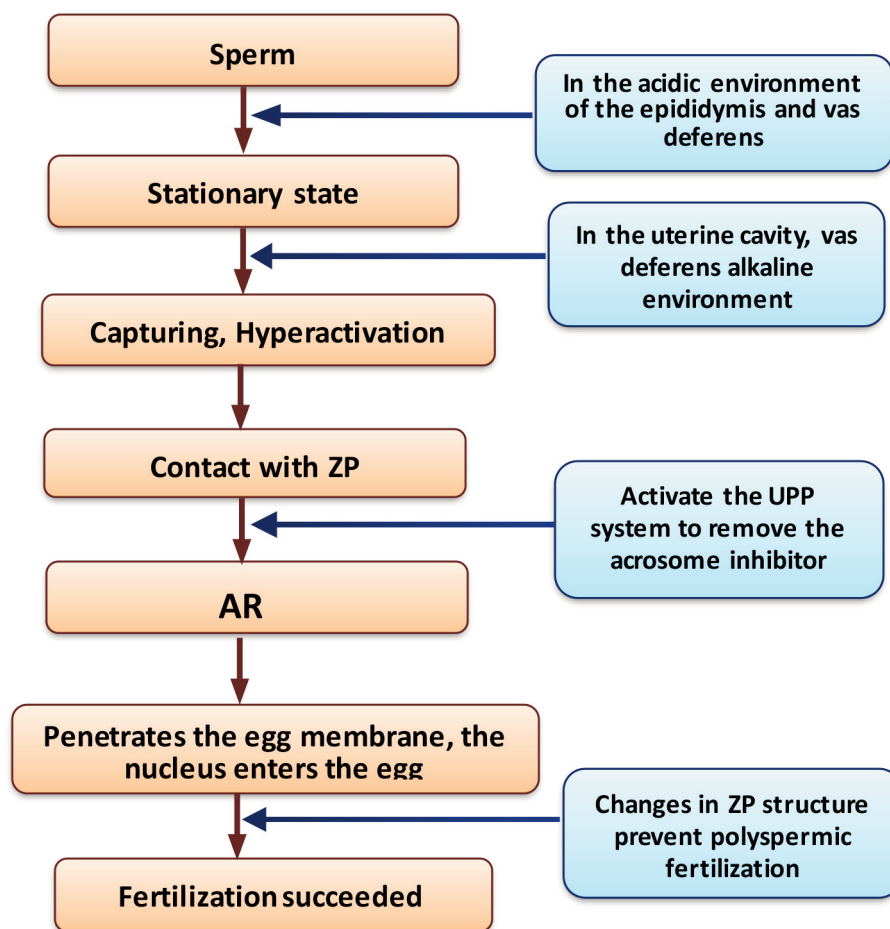
Figure 1. *Schematic diagram of the fertilization process. A schematic diagram of the fertilization process of sperm from the epididymis, vas deferens, to the uterine cavity and fallopian tubes with changes in the environment, which includes the activation of the UPP system after combining with ZP, the removal of the acrosome inhibitory factors, and the sperm nucleus entering the egg to cause ZP. The process by which structural changes prevent polyspermy. UPP: Ubiquitin proteasome pathway; ZP: zona pellucida.*

changes in the sperm membrane, and protein phosphorylation. When sperm pass through the cervix, a large number of de-energizing factors are removed, and the permeability and fluidity of the cell membrane increase, allowing regulatory molecules such as Ca^{2+} and HCO_3^- to enter the cell. Activation of adenylate cyclase and an increase in cAMP are core events in the molecular mechanism regulating sperm capacitation. cAMP can activate downstream signaling pathways, including the very important protein kinase A (PKA) pathway that is involved in protein tyrosine phosphorylation. Sperm entering the uterus sense changes in voltage, ligands, phosphorylation, second messengers, and other signals through the action of ion channels or transporters on the sperm membrane, resulting in changes in the conformation of sperm membrane proteins that generate ion flow. Thus, fertilization is regulated by changes in intracellular and extracellular ion concentrations. After the capacitated sperm and the egg meet in the ampulla of the

fallopian tube and the sperm contacts the corona radiata of the egg, hyperactivation occurs, allowing the sperm to overcome resistance and contact the zona pellucida (ZP). The instability of the sperm membrane increases after the acrosome contacts cumulus cells and leads to the removal of the inhibitory roof. The acrosome reaction (AR) refers to a series of changes in the acrosome that expose the ZP receptor on the sperm surface. The AR is a receptor-mediated cell exocytosis process, in which the ZP receptor binds to ZP3, a ZP glycoprotein, phosphorylates tyrosine protein kinase and activates G protein. Then, cholesterol effluxes, the plasma membrane becomes liquefied, and ZP receptors move within the lipid bilayer membrane, inducing fusion of the sperm plasma membrane with the preacrosomal membrane to form a channel that exposes the nucleus *via* the anterior acrosomal inner membrane (1). Acrosome vesicles release acrosome proteases and other substances to hydrolyze the ZP around the oocyte and form a

channel that allows only one sperm can pass through, further promoting oocyte activation. The AR is irreversible due to changes in sperm cell membrane proteins, the rupture and loss of acrosomes, and permanent changes in sperm morphology. To further explore the influencing factors and regulatory mechanisms underlying the fertilization process, research on the precise regulatory mechanisms of fertilization is reviewed (Figure 1).

Factors Triggering and Influencing Acrosome Reaction

The acrosome is a membranous organelle at the sperm head; it covers the front 2/3 of the sperm head and is separate from the nucleus. This structure consists of vesicles and contains a variety of hydrolytic and digestive enzymes related to fertilization. Acrosomal contents are stored in a highly concentrated form in the acrosome for period up to several weeks, from sperm formation in the testis to maturation in the epididymis. The outer acrosomal membrane has a dense structure consisting of three macromolecular glycoproteins that stabilize the membrane, bind to lectins, and play a role in fusion with the plasma membrane during the AR. The acrosome is divided into pre-acrosomal and equatorial regions. The posterior ring of the acrosome is adjacent to the equatorial part of the acrosome. The caudal region under the membrane is the egg-recognition site. During fertilization, the plasma membrane covering this region first fuses with the egg's plasma membrane, and the absence of the posterior ring of the acrosome can lead to infertility. The acrosome plays a decisive role in egg penetration. The AR is an important indicator for evaluating sperm function during fertilization. In addition to intracytoplasmic sperm injection (ICSI), *in vivo* fertilization and *in vitro* fertilization (IVF) require the sperm AR to support contact, fusion, and fertilization between the sperm and egg (2). Completion of the AR is marked by complete fusion of the outer acrosomal membrane with the sperm cell membrane. Acrosomal enzymes include hyaluronidase, corona radiata dispase, acrosomal serine protease, and aromatic sulfatase. Serine protease and hyaluronidase are activated through the AR after capacitation and play roles in dissolving and penetrating the ovum corona radiata and ZP. Therefore, they are key enzymes supporting the fusion of sperm and egg cells. Acrosomal activity can accurately reflect acrosome function. A low acrosome integrity rate indicates that acrosomal activity is insufficient, which affects decomposition of the cumulus, penetration of the ZP, and eventually, sperm fertilization capacity (3). Acrosomal enzymes can also affect sperm motility. Abnormal sperm cells with the damaged acrosomes are unable to get through the outer membrane of an egg cell to fertilize it, leading to infertility in affected men. Sperm having a defective acrosome cannot bind to the ZP and vitelline membrane and must rely instead on assisted reproductive technology to achieve reproductive success. Clinically, the intensity of acrosomal enzyme activity can be predicted through examination of the sperm acrosome integrity rate. It has been suggested that the acrosome integrity rate of sperm is positively correlated with the activity of acrosomal enzymes. However, these inferences are not conclusive, as accurate determination of the activity of an enzyme requires comprehensive analysis, including the time of the enzymatic reaction and the rate of the enzymatic activity.

Oval ZP glycoprotein, progesterone (P4), follicular fluid, gamma-aminobutyric acid (GABA), and calcium ionophore A23187 are among the substances that can trigger the AR. ZP glycoprotein induces the AR in human sperm, which is essential for sperm passage through the ZP. Progesterone, present in the follicular fluid and fallopian tube fluid, is a natural inducer of the AR. Follicular fluid, whose main active ingredient is progesterone, can induce AR by promoting the influx of calcium ions from the area outside the capacitated sperm cells. While GABA induces the AR through activation of sperm plasma membrane surface receptors, leading to an influx of calcium ions that surround human sperm cells. Ca^{2+} and energy are important factors for sperm capacitation and the AR. Studies have found that a certain amount of tumor necrosis factor alpha (TNF-α) can reduce the activity of Ca^{2+}-ATPase and alter the Ca^{2+} concentration in sperm, which may inhibit acrosomal enzyme activity and the AR. At the same time, TNF-α can significantly reduce the activity of superoxide dismutase in sperm, weakening the ability of sperm to scavenge oxygen free radicals and thus increasing the toxicity caused by oxygen free radicals. This process inhibits acrosomal enzymes and decreases AR. A previous study found that the reduction of sperm fertilization capacity caused by interleukin (IL)-2 may be driven by its strong inhibitory effect on acrosomal enzymes. Treatment of human sperm with IL-6 at concentrations greater than or equal to 0.5 ng/ml significantly increased the acrosomal activity of human sperm, and the AR rate was significantly enhanced in a dose-dependent manner, suggesting that increased acrosomal enzyme activity may be an inducer of sperm AR. Nitric oxide (NO) plays dual roles in the male reproductive system. Low concentrations of NO can protect sperm motility, whereas high concentrations inhibit sperm motility and reduce sperm AR rate. GABA is an inhibitory transmitter that regulates the secretion and release of pituitary gonadotropins by promoting the secretion of gonadotropin-releasing hormone in the hypothalamus. Male gonads and accessory gonads are also closely associated with sperm motility and steroid hormone production. Through evaluation of acrosomal enzymes in normal male sperm and anti-sperm antibody-positive sperm, Shuling Bian *et al*. found that GABA can significantly increase acrosomal enzyme activity in both sperm types ($p<0.01$). This effect can be mediated through GABA receptors that are present on the membrane of human sperm.

Degradation of Acrosome Inhibitors *via* the Ubiquitin-proteasome Pathway

Acrosome inhibitors can inhibit the activity of acrosomes in sperm, mainly in the epididymis *via* changes in the acrosomal area on the surface of ejaculated sperm. Sperm generally exhibit acrosomal activity during capacitation and the AR. Acrosomal enzymes are activated only when the AR occurs (4). Prior to sperm capacitation or the AR, acrosome inhibitors impede acrosomal enzyme activity and prevent sperm damage before capacitation. However, capacitation exposes acrosomal enzymes, and this process requires the ubiquitin-proteasome pathway (UPP) to degrade acrosome inhibitors and thus prevent their activity. The UPP is an efficient and specific ATP-dependent protein degradation system that operates in the cytoplasm and nucleus. The UPP begins with the activation of ubiquitin (Ub) that is affected by ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2, UbE2), ubiquitin ligase (UbE3), and deubiquitinating enzyme. In a cascade process regulated by deubiquitylating enzymes (DUBs), Ub-tagged substrate proteins interact with the 26S proteasome complex for degradation into oligopeptides. The UPP is involved in sperm capacitation and the AR, promotes sperm acrosome exocytosis, degrades ZP protein when the sperm and egg combine, assists sperm penetration of the ZP, and promotes the fusion of sperm with the ZP during the process of IVF. Activation of the 26S proteasome is essential for sperm capacitation and acrosome exocytosis. 26S proteasome activity occurs within and on the surface of mammalian sperm acrosomes, triggering post-AR exocytosis upon sperm–egg interaction. The 26S proteasome is located in the acrosomal inner membrane after acrosome exocytosis, and may promote sperm–egg interaction and provide stable proteolytic enzyme activity while the sperm head passes through the ZP. Ubiquitinated proteins are present in the mammalian oocyte ZP, which inhibits the hydrolytic activity of 26S to hinder fertilization by altering sperm capacitation, acrosome exocytosis, and sperm binding to oocyte ZP.

After sperm capacitation, the plasma membrane undergoes major changes. Initially, the internal pH of the acrosome is acidic, and sperm preacronin is inactive. The pH increases after sperm capacitation due to proteolysis of preacronin; this change activates sperm enzymes, which then initiate the hydrolysis of acrosome components through the 26S proteasome. Mengmeng Li's research shows that acrosome inhibitors are ubiquitinated with four ubiquitin molecules after capacitation or the AR, and they are completely degraded by the ubiquitin system. In conclusion, the stable and successful release of the sperm acrosome requires removal of acrosome inhibitors. The presence of acrosome inhibitors on the sperm surface plays a crucial role in regulating binding of sperm to the ZP.

Effects of Sperm Morphology and Motility on the Fertilization Process

The morphology of sperm reflects the contents of the sperm acrosome to a certain extent. Studies have shown that the activity of the sperm acrosome is positively correlated with sperm motility, semen concentration, and normal sperm morphology (5). Cervical mucus restricts the penetration of morphologically abnormal sperm, as sperm with abnormal heads may experience greater resistance from cervical mucus and severely deformed and elongated sperm cannot bind to the ZP. Thus, a normal acrosomal morphology is more critical than the normal morphology of the sperm itself, as a round-headed sperm lacking an acrosome is unable to combine with the egg's ZP and vitelline membrane. Sperm with mild abnormalities such as a small oval shape can bind to the ZP if the acrosome is normal, but this binding is slightly weaker than that of normal sperm. The acrosome size can influence the occurrence of a spontaneous AR, as sperm with large and normal acrosomes have higher rates of a spontaneous AR than small-acrosome sperm (6). When morphologically normal sperm comprise ≥15% of the total sperm, the incidence of a ZP-induced AR significantly increases. The proportions and morphology of the sperm head acrosome are important indicators used in sperm morphological analysis, as most abnormalities of sperm morphology are due to an abnormal acrosome. A significant negative correlation was observed between acrosomal contents and the acrosome ratio, implying that the contents of the sperm acrosome decrease with an increasing acrosome proportion. This finding suggests the presence of components that inhibit the formation of the sperm acrosome in the cytoplasm of sperm during spermatogenesis, which balance excessive production of sperm acrosomes and prevents sperm autolysis due to excessive acrosome levels. For individuals with severe teratozoospermia, ICSI is the best option for conception. In the process of ICSI, if an abnormal sperm is injected into the egg cell, the chromosome aneuploidy rate of the abnormal sperm can be high, which not only reduces the fertilization rate but also transfers an abnormal genome to the oocyte, resulting in abnormal embryo development. DeVos *et al.* compared the oocyte fertilization rate, embryo morphology, embryo cleavage quality, and post-implantation pregnancy rate after ICSI between morphologically abnormal sperm and morphologically normal sperm. They found that sperm morphology can affect the results of fertilization after ICSI, but not embryo development. However, Check *et al.* found that abnormal sperm morphology did not affect ICSI results. Berkovitz *et al.* demonstrated that the embryo implantation rate and pregnancy rate after ICSI using spermatozoa with normal nuclear morphology were significantly higher than the corresponding values in the group with abnormal nuclear

morphology, and the abortion rate was significantly lower. However, some sperm with abnormal nuclear morphologies can still be fertile through ICSI. The formation of the sperm nucleus is a fragile process during spermatogenesis. At this time, histones are gradually separated from DNA and replaced with transition proteins and protamines. Chromatin is easily damaged due to loss of its protection, resulting in sperm deformities. Sperm motility is an important function of normal mature sperm, as it ensures that the sperm and egg meet, plays a role in the mechanical penetration of the egg, and is one of the main determinants of fertility. Asthenozoospermia hinders sperm passage through the cervical canal and uterine cavity to the fallopian tube cavity and combination with the egg, resulting in fertility disorders. Sperm motility is closely related to sperm morphology. Rigorous evaluation of sperm morphology and the mean sperm velocity provide predictions of fertilization capacity. When a certain proportion of normally shaped sperm in total sperm are present, normal motility and viability are assured. Malformed sperm, especially sperm with head deformations, such as large-headed sperm, encounter increased resistance during swimming, which reduces swimming speed. Sperm with vacuolar abnormalities of the head are unstable. Such deformed sperm cannot reach the fallopian tube and contact the egg. The sperm neck, middle, and tail are the locations of the mitochondrial sheath and axoneme, which are structures important for sperm energy supply and motility, respectively. Hyperactivation is an important prerequisite for sperm capacitation and oocyte penetration and fusion and is closely related to sperm morphology. The proportions of sperm with a normal head morphology and a small acrosome were found to be significantly higher among hyperactivated motile spermatozoa, whereas the proportions of spermatozoa with large and round heads and spermatozoa with defective midsections and tails were significantly decreased. The sperm flagellum is an important sperm motility organ. Defective flagella can lead to low sperm motility, which in turn affects the transmission of energy. Patients with asthenozoospermia often also exhibit sperm with flagella deformities. The flagellum is an important subcellular structure of spermatozoa, and whether abnormal flagellar morphology affects sperm motility remains under debate.

Mechanisms Underlying Polyspermia Prevention

The average human body produces 100 million sperm per ejaculation, but only a small number of those sperm approach the egg, and only one sperm combines with the egg. This selectivity occurs because the fertilized egg can alter the structure of the oocyte plasma membrane to block polyspermic fertilization. Adding an appropriate amount of hyaluronidase inhibitors (such as tannic acid) to animal IVF medium can reduce the polyspermic fertilization rate without

affecting sperm penetration, indicating that hyaluronidase plays an important role in the fertilization process (7). Injection of phospholipase C-ζ into oocytes has been reported to activate the oocytes. After oocyte activation, cortical granules release enzymes that alter the structure of and harden the ZP, thereby effectively preventing polyspermic fertilization. Changes in intracellular calcium ion concentration related to oocyte activation affect cell membrane blockade, but these conditions are not sufficient to ensure blockade. Romar *et al*. found that calreticulin was released after oocyte activation and was associated with cell membrane blockade. The ZP is the main structure that blocks polyspermic fertilization. After fertilization, cortical granules promote the reorganization of cytoskeleton-related proteins such as actin, filamentous actin, and myristoylated alanine-rich C kinase substrate (MARCKS) through the action of Ca^{2+}-dependent proteins, leading to the exocytosis of cortical granules. Cortical granules contain enzymes such as proteolytic enzymes, glycosidases, and cross-linking enzymes, which can promote structural changes in the ZP receptors ZP2 and ZP3. After fertilization, ZP2 undergoes proteolytic cleavage through oocyte-specific lecithin (a metalloprotease), leading to changes in the supramolecular structure of the ZP that surrounds the oocyte, and the change in ZP3 structure causes ZP to lose its sperm recognition and binding capacity, thereby preventing free sperm from attaching to the ZP. ZP2 is proteolytically hydrolyzed to ZP2f, which prevents penetration of ZP by sperm that have already contacted the ZP, preventing excess sperm penetration. The ZP surface of the unfertilized egg is reticulate and becomes smooth and less porous after fertilization. The protein filaments that constitute ZP decrease in length and increase in thickness, making the ZP thicker and stiffer. F-actin forms a dynamic network under the oocyte plasma membrane and binds to MARCKS in nonactivated oocytes; this cytoskeleton acts as a barrier and prevents cortical granule discharge prior to the activation of secondary oocytes. After activation, MARCKS dissociates from F-actin, thereby initiating cortical granule exocytosis and the release of contents into the periovular space.

Regulation of Fertilization Through the cAMP-PKA Signaling Pathway of Progesterone and Tyrosine Phosphorylation

Follicular fluid contains numerous hormones including follicle-stimulating hormone and sex hormone, mucopolysaccharides, serum protein, and plasma exudate. The main hormone is progesterone, which is secreted by the ovaries in the human body and an essential component of the reproductive cycle. Some hormones transfer information about various functions that may lead to the stopping or starting of those processes (8). Increased protein phosphorylation is associated with

capacitation, sperm ZP binding, and the AR, whereas sperm ZP binding and fertilization capacity were found to decrease after treatment with anti-phosphotyrosine monoclonal antibodies. cAMP-PKA is a second messenger system involved in non-genomic processes regulated by steroid hormones. The intracellular cAMP content and activation of PKA lead to phosphorylation of serine, threonine, and tyrosine in sperm proteins, which regulate various physiological processes including sperm capacitation, hyperactivation, chemotaxis, and the AR. cAMP can enhance the motility of hyperactivated sperm. The attenuation of various signal transduction pathways can affect tyrosine phosphorylation and reduce the importance of sperm surface proteins and ZP proteins. Capacitated sperm initiates the AR in the female reproductive tract through regulation of a signaling pathway involving protein phosphorylation at tyrosine residues. Mitogen-activated protein kinases are dual-specificity kinases that can phosphorylate serine/threonine and tyrosine, and the extracellular signal-regulated kinase (ERK) of the mitogen-activated protein kinase (MAPK) pathway induces tyrosine phosphorylation of proteins on the sperm surface. CatSper is a calcium ion channel that specifically controls protein tyrosine phosphorylation in intact mouse sperm in a time-dependent manner. Progesterone is regulated through non-genomic processes mediated by membrane receptors, and Ca^{2+} pools and ion channels or transporters on sperm cell membranes play important roles in the regulation of hyperactivation (9). Progesterone regulates sperm–egg binding by activating CatSper on the surface of human and mouse sperm, which instantaneously increases the contents of intracellular Ca^{2+} ions and cAMP, leading to sperm capacitation, hyperactivation, chemotaxis, the AR, and other physiological functions (10).

α/β Hydrolase 2 (ABHD2) is the central protein responsible for the non-genomic effects of progesterone on sperm target proteins. Transcription and expression of ABHD2 differ between clinically infertile and normal reproductive male sperm, and progesterone regulates sperm fertilization through the ABHD2 receptor-mediated cAMP-PKA signaling pathway. Progesterone activates CatSper in human sperm through ABHD2, thereby strongly stimulating the main Ca^{2+} ion channel on sperm flagella (11). Progesterone can also modulate CatSper through competing with other hormones (*e.g.*, testosterone and hydrocortisone) (12). After interaction with progesterone, ABHD2 is activated to decompose triacylglycerol fat and generate diacylglycerol (DAG). The inhibitor monoacylglycerol (MAG)/DAG of CatSper is converted to allow activation of CatSper, downstream signals, and physiological functions such as chemotaxis and the AR, as well as Ca^{2+} influx and sperm capacitation and hyperactivation. The same study demonstrated that the binding of progesterone to ABHD2 protein affects the regulation of the cAMP-PKA signaling pathway; this pathway can interfere with the binding of progesterone to ABHD2 protein, blocking CatSper. CatSper can prevent the active movement of sperm and cause them to return to an inactive state without affecting their survival under natural conditions, that is, sperm cells experience no toxic side effects and can still swim but have insufficient power to penetrate egg cells (13). Comparison of the expression of ABHD2 at the mRNA and protein levels between a normal fertility group, oligoasthenozoospermia group, and AR abnormality group shows that the expression level is significantly higher in the AR abnormality group than in the oligoasthenozoospermia group. The discovery of ABHD2 explains some cases of infertility, and development of a method to block ABHD2 could provide a novel means of contraception.

Regulation of Fertilization by Calcium, Calmodulin, and the cAMP-PKA Pathway

Calcium ion influx plays an important role in sperm capacitation, the AR, sperm chemotaxis, and membrane fusion. Obstruction or deletion of calcium ion channels can lead to infertility. Studies have shown that calcium ion concentrations of 1.0 μmol/l can improve sperm capacitation, whereas spontaneous ARs occur at lower levels. The same study demonstrated that an increase in cytosolic Ca^{2+} alone is insufficient to trigger the AR, the action of intra-acrosomal Ca^{2+} is mediated by IP3 receptors and sufficient levels of extracellular Ca^{2+} are also required to trigger the AR that occurs after sperm capacitation. High levels of calmodulin (CaM) are present in the head and tail of mammalian sperm (14). CaM acts as a calcium ion sensor in cells. If purified CaM is added to capacitation solution at 10 μmol/l, the overall rate of sperm capacitation is not affected, but the formation of the sperm membrane is significantly accelerated with CaM inhibitor addition to the capacitation fluid. In this process, the tyrosine phosphorylation level of sperm capacitation protein, motility of sperm, and formation of the sperm membrane are significantly inhibited, indicating that CaM plays important roles in sperm membrane formation and the AR during capacitation. CaM can stimulate the activity of adenylyl cyclases (ACs), thereby increasing the concentration of intracellular cAMP, and can also regulate sperm protein tyrosine phosphorylation and hyperactivation through stimulation of ACs. The sperm acrosome protein IQCF1 is specifically expressed on testicular sperm cells and mature sperm acrosomes. IQCF1 is closely associated with the levels of tyrosine phosphorylation during capacitation and the AR due to its binding to CaM. IQCF1-knockout male mice exhibited decreased sperm motility and AR rate. During capacitation, the pH in the female reproductive tract increases, and large quantities of K^+ efflux from sperm, accompanied by influx of large quantities of extracellular Ca^{2+}, HCO_3^-, and Na^+. The influx of HCO_3^- and Ca^{2+} induces a series of signaling

pathways in sperm. Activation of ACs can increase the intracellular cAMP concentration, which in turn stimulates the activity of PKA and other kinases. Phosphorylation of some sperm proteins occurs at serine, threonine, and especially tyrosine sites, resulting in sperm hyperactivation and the AR. Studies have shown that HCO_3^-, Ca^{2+}, and bovine serum albumin (BSA) lead to tyrosine phosphorylation, mediating sperm capacitation. An increase in the calcium ion concentration activates phospholipase A2, which digests fatty acids from phospholipids, promotes the fusion of the two membranes at numerous points to form vesicles, and releases acrosomal contents at the sperm–egg binding site. These processes lead to digestion of the ZP. When AR occurs, the internal Ca^{2+} levels and pH value of sperm cell increase, and Ca^{2+} activates phospholipase C (PLC-β), which, after being stimulated by G protein, hydrolyzes phosphatidylinositol on the membrane to produce diglyceride and phosphoinositide. Diglyceride activates phospholipase A2, which generates lysophospholipids that cause fusion of the outer acrosome with the plasma membrane. When IP3Rs are stimulated by the intracellular second messenger IP3, the stored Ca^{2+} is released. This release directly or indirectly causes the influx of exogenous Ca^{2+} and triggers protein tyrosine phosphorylation, leading to exocytosis. The increased Ca^{2+} concentration activates the phosphatidylinositol signaling pathway, which in turn activates protein kinase C (PKC). PKC opens voltage-dependent Ca^{2+} channels on the sperm plasma membrane, causing a large influx of Ca^{2+}. Actin depolymerization between the acrosome membrane and plasma membrane leads to their contact, which in turn promotes the AR. Although Ca^{2+} is distributed on the outer surface of the outer acrosome and the inner surface of the plasma membrane, and exogenous Ca^{2+} is also necessary for the AR.

The presence of tyrosine-phosphorylated proteins and acrosome-binding protein (ACRBP) on the surface of sperm (15) induces capacitation and initiates the sperm AR by regulating intracellular calcium through the muscle/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump. SERCA is a calcium ion transporter mainly located in the endoplasmic reticulum and sarcoplasmic reticulum, and it transports calcium ions from cytoplasm against a concentration gradient into the endoplasmic reticulum. ACRBP is a secondary ZP-binding affinity protein that is transported to the surface of the acrosome in intact sperm during capacitation and interacts with other proteins on the sperm surface to allow sperm penetration of the egg through the AR. Addition of anti-ACRBP antibody to sperm pre-culture medium before fertilization reduces capacitation, the AR, sperm ZP-binding capacity, and the fertilization rate. These effects occur because anti-ACRBP antibody localizes to the sperm head, which reduces the capacity of sperm to initiate the AR in response to proteolysis of the ZP or inhibition of SERCA, thereby preventing a thapsigargin-induced and soluble ZP-induced AR. During capacitation, the calcium concentration is

significantly increased *via* several calcium channels, including voltage-gated Ca^{2+} channels, CatSper, and transient receptor potential (TRP) channels, which are often described as cation channels that increase intracellular Ca^{2+} levels (16). These channels are activated by a variety of stimuli, including changes in temperature, pH, and osmotic pressure, and are regulated through growth factor and G protein-coupled receptor pathways. TRP channels regulate major pathways involved in sperm capacitation, with capacitation enhancing the translocation of TRP from the post-acrosomal region to the apical region of the sperm head. Activation of phospholipase C triggers the opening of TRP channels and the release of Ca^{2+} from storage. After sperm enter the alkaline female reproductive tract, the increased intracellular pH activates sperm membrane CatSper, increasing the intracellular Ca^{2+} concentration and inducing sperm hyperactivation (17). The environmental pollutant bisphenol A (BPA) can significantly inhibit the motility and AR of mouse sperm, as well as CatSper, suggesting that BPA may damage sperm function in mice through CatSper. Progesterone, as well as various physiological substances such as bourgeonal, mediate Ca^{2+} influx to human sperm through CatSper (18). CatSper-mediated Ca^{2+} influx can be activated by the environmental pollutant p,p'-dichlorodiphenyldichloroethylene (19). ACRBP directly or indirectly activates Ca^{2+} channels, such as TRP, through the SERCA pump, resulting in the storage of calcium ions in sperm, and anti-ACRBP antibodies may affect capacitation and the AR through their effects on Ca^{2+} channels. Thapsigargin is a non-competitive inhibitor of the SERCA pump that increases the intracellular Ca^{2+} concentration in sperm and induces the AR, resulting in 10–300-fold higher Ca^{2+} concentrations relative to somatic cells. Addition of thapsigargin to human spermatozoa causes a dose-dependent increase in the percentage of sperm undergoing the AR in the presence of calcium, as well as in cases of progesterone- and thapsigargin-induced ARs, due to Ca^{2+} influx from the extracellular medium through storage-operated Ca^{2+} channels (20). The calcium ionophore A23187 is an artificial agonist that forms a lipid-soluble complex with calcium ions, leading to the rapid influx of calcium ions through the sperm plasma membrane, which in turn causes the fusion of the sperm plasma membrane with the outer acrosome, resulting in the AR. A23187 induces the AR in sperm treated with anti-ACRBP antibody, effectively preventing thapsigargin-induced AR. Studies have shown that recombinant ZP3 enhances calcium efflux from intracellular Ca^{2+} stores at the onset of the AR in several mouse, porcine, and bovine models. ZP3-activated receptors induce sperm membrane depolarization through low-voltage-activated Ca^{2+} channels and activated phospholipase C; this process involves IP3-activated IP3 receptors on the acrosome. The release of Ca^{2+} from intracellular stores generates a signal that activates TRP channels in the plasma membrane. Pre-acrosomal interactions of zonulin and ACRBP may shift from the acrosomal region to the sperm surface. Ribbon-like mucin in the anterior plasma

membrane of the sperm head interacts with ZP3, which is also involved in sperm binding to the ZP during capacitation (21). An increase in cytosolic calcium levels may be caused by the inhibition of SERCA by thapsigargin, whereas activation of SERCA by gingerol may reduce the cytoplasmic calcium concentration, leading to greater Ca^{2+} sequestration in the acrosome and delayed AR, which is consistent with the results of thapsigargin treatment. The percentage of spermatozoa undergoing the AR was higher in spermatozoa treated with thapsigargin than in those cultured in capacitation medium with no additions. In a comparison of spermatozoa cultured with gingerol or BAPTA (1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid)-K^+, the presence of gingerol resulted in a smaller percentage of sperm undergoing the AR compared to sperm treated with thapsigargin.

Fertilization Regulatory Mechanisms Involving the KSper Channel, Na Bicarbonate Cotransporter, and Na+/H+ Exchanger

The sperm-specific KSper channel is the main K^+ channel in mammalian sperm. KSper is a strongly pH-sensitive and voltage-dependent potassium ion channel with high conductance that is closely associated with sperm motility, membrane hyperpolarization, and the AR (22). Deletion of either KSper or the sperm-specific Ca^{2+} channel results in complete male sterility (23). Na bicarbonate cotransporter (NBC) is an exchanger of Na^+ and HCO_3^- that regulates intracellular alkalinity by transporting HCO_3^-, a weakly basic ion. Na^+/H^+ exchanger (NHE) is an exchanger of Na^+ and H^+ that regulates intracellular pH by transporting H^+ and is an important regulator of the intracellular pH of sperm (24). NHE is expressed in both the testis and sperm midsection, and antibodies against testis-specific NHE reduced intracellular pH, sperm AR rate, and sperm motility. The KSper channel in sperm plays a decisive role in regulating the membrane potential of sperm during hyperpolarization, and the increase in sperm intracellular pH is a key factor affecting the activation of KSper. An NHE inhibitor with no effect on KSper, DMA (5-(N,N-dimethyl)amiloride hydrochloride), reduces intracellular pH through the inhibition of the NHE transporter, which in turn inhibits KSper channel opening, leading to depolarization of the mouse sperm membrane potential. During the capacitation of mouse sperm, NHE opens the KSper ion channel by sensing the increase in extracellular pH, resulting in hyperpolarization of the sperm membrane potential. Further opening of NHE, which increases intracellular pH, leads to further opening of KSper, and this feedback between NHE and KSper protein leads to sperm membrane potential hyperpolarization and the intracellular pH change necessary for CatSper-mediated calcium influx (25). The increases in intracellular pH and Ca^{2+}, and hyperpolarization of spermatozoa membranes are important components of the AR in spermatogenesis. NHE regulates the KSper ion channel through regulation of pH, and the NHE inhibitor DMA reduces the intracellular pH of mouse sperm by inhibiting the NHE transporter. Thus, the membrane potential mediated by KSper in mouse sperm is depolarized, with an inhibitory effect on the AR (19).

Roles of the Zona Pellucida (ZP) and ZP Proteins in Egg Fertilization

The direct cause of AR induction during spermatogenesis is contact with the ZP on the egg surface, specifically with ZP3. ZP3 can bind directly to sperm and becomes inactive after fertilization. After a capacitated sperm is exposed to ZP, numerous receptors on the sperm cell membrane specifically recognize and bind to the ligand ZP3, thereby activating sperm calcium ion channels and resulting in a large influx of calcium ions and elevated concentrations of other second messengers, such as cAMP and IP3. The instantaneous increases in the calcium ion and cAMP concentrations activate certain protein kinases, as well as increase sperm pH, causing actin between the sperm cell membrane and acrosomal membrane to begin depolymerizing into monomers. The barrier between the membranes disappears and the distance between the two membranes becomes very small, facilitating membrane fusion (26, 27). Human ZP comprises a unique extracellular matrix material around eggs; it is a fine mesh structure consisting of interconnected filaments that surround the extracellular glycoprotein shell of the human oocyte. The ZP exhibits strong selectivity for sperm morphology. Larger sperm with good bilateral symmetry and no deformity in the neck are more likely to combine with the ZP. The ZP, corona radiata, and cumulus form the oocyte–cumulus–corona radiata complex (28). The ZP consists of four glycoproteins, ZP1, ZP2, ZP3, and ZP4. ZP filaments are cross-linked by ZP1 homodimers to form a stable matrix (29). The multilayered structure of the ZP forms an envelope that supports the blastomere, helps to avoid inappropriate and premature implantation in the fallopian tube, and protects the embryo from the maternal immune system. N-linked glycosylation of ribbon proteins plays an important role in the induction of AR. Both glycans and protein–protein interactions are involved in human gamete interactions. ZP glycoproteins play key roles in the processes of oogenesis, taxon-specific binding of sperm to oocytes, and induction of the AR when sperm bind to the ZP. The ZP matrix functions as a gatekeeper that regulates sperm binding. ZP glycoprotein also prevents polyspermic fertilization. The ZP matrix protects the growing embryo prior to implantation. Deficiencies in the ZP play an important role in fertilization failure during conventional IVF. Mutations in genes encoding human ZP glycoproteins can cause an abnormal ZP matrix and female infertility. Studies have shown that oocytes from

mice heterozygous for such mutations have a thinner ZP, and female mouse oocytes with two mutations have a thinner ZP than oocytes with a single mutation or those lacking the ZP entirely, suggesting that the mutation has a negative effect on the ZP (30). These mutations have been shown to cause defects in expression, secretion, and interactions that lead to abnormal oocyte phenotypes in patients (31). Co-immunoprecipitation and homology modeling analyses have demonstrated that mutant ZP1 disrupts oocyte ZP formation through interference with the interaction between ZP1, ZP2, and ZP3 (32). Immunofluorescence and histological analyses revealed that some degenerated oocytes lack ZP stroma (33).

ZP1 has a structural role, whereas ZP2 and ZP3 function as primary and secondary receptors. Analysis of the *ZP* gene reveals that the rate of sequence variation in the *ZP1* and *ZP3* genes is 1.5-fold higher in the eggs of women who could not achieve fertilization through IVF than women with normal IVF fertilization results. N-linked glycans of human ZP proteins induce the AR more readily than do O-linked glycans. Using eggs from transgenic mouse lines expressing human ZP1, ZP2, ZP3, and ZP4, researchers found that ZP2 plays an important role in human sperm–egg binding, whereas ZP1, ZP3, and ZP4 may not be involved in this process. By contrast, using purified native and recombinant proteins, human ZP1, ZP3, and ZP4 have been shown to bind to capacitated human sperm and induce the AR. ZP1, ZP2, and ZP3 play roles in fertility and ZP matrix assembly.

Sperm–Egg Union

Fertilization times can be divided into ultra-short fertilization (less than 30 min), short fertilization (1-6 h), and overnight fertilization (16-18 h). The fertilization process is regulated by precise molecular mechanisms. Mammals employ three distinct membrane fusion processes to complete fertilization. First, sperm cells secrete acrosomal inclusions, which allow sperm to penetrate the oocyte's ZP to reach the oocyte plasma membrane, the site of fertilization. Next, the sperm cells combine and fuse with the oocyte plasma membrane (also known as the egg membrane) (34). Finally, protein molecules involved in the fertilization process, namely izumo sperm-egg fusion 1 (IZUMO1) on the sperm and CD9 on the egg, are critical to sperm–egg fusion. CD9 is a four-transmembrane domain protein expressed on eggs that plays a key role in sperm–egg fusion. Studies have shown that eggs lacking CD9 expression cannot fuse with sperm normally. The length, thickness, and density of microcilia on eggs can vary, but the CD9 gene is needed to support healthy male and female gametes in mice. IZUMO1 is involved in sperm–egg plasma membrane fusion in mammals. A glycosylated IZUMO1 is abundantly expressed in the mouse testis, and its concentration decreases sharply after sperm enter the epididymis. This finding shows that the glycosylation site is important for

stabilizing the structure of the IZUMO1 protein. Even if the CD9 protein is not expressed on the egg surface, the functional domain of IZUMO1 can bind to the egg membrane. IZUMO1 plays a mediating role in the sperm–egg plasma membrane fusion process, and the active site is the helical structure at the N terminus (35). IZUMO1 is located on the acrosomal cap of the sperm head; after the AR, IZUMO1 moves toward the equatorial region of the sperm, where initial sperm–egg fusion occurs. Spermatozoin is involved in sperm–egg fusion, and PH-30 has an inhibitory effect on this process. The antigens recognized by PH-30 include spermatin a (Adam 1b) and spermatin p (Adam 2). Knockout of the *Adam 2* gene in sperm blocks the fertilization pathway, and the sperm loses its capacity to pass through the ZP, resulting in infertility stemming from the prevention of sperm–egg fusion. Blockade of the *Adam 1b* gene, associated with fertilizin, allows sperm–egg fusion to occur in the absence of fertilizin. In humans, *Adam 1b* is a pseudogene, but in sperm within the mouse testis, Adam 1b and Adam 2 are distributed on the cell membrane of the sperm head, and after sperm enter the epididymis, fertilizin is transferred to the back half of the head. Human CD46 is a widely expressed protein that protects the host cell from damage by complement. CD46 is involved in sperm–egg binding and mediates sperm–egg fusion. Various anti-human CD46 monoclonal antibodies can inhibit the fertilization process. *In vitro* or *in vivo* fertilization experiments using mice without CD46 expression mutations show that the sperms are prone to spontaneous AR. In addition, *in vitro* experiments show that anti-PDIA3 antibody inhibits sperm–egg fusion; thus, PDIA3 may play an important role in sperm–egg fusion. During sperm–egg union, sperm CRISP1 and CRISP2 bind to the fusion structure of the egg. Anti-CRISP1 and anti-CRISP2 antibodies can effectively inhibit the fertilization process, although CRISP1 is not required for fertilization.

Summary and Prospects

The process of fertilization depends upon the shape and vitality of the sperm, especially the shape of the acrosome, which plays a decisive role in fertilization. Sperm remain quiescent in the acidic environment of the epididymis and vas deferens, whereas the alkaline environments of the uterine cavity and ureter lead to sperm capacitation, hyperactivation, fusion with the egg, the AR, and the release of acrosome enzymes to complete the fertilization process. Changes in pH in the sperm's external environment, the level of progesterone in the uterine cavity, calcium ions, calcium channels and related signaling pathways, and structural changes in the ZP surrounding the egg play crucial roles in fertilization. At the same time, a system for controlling acrosomal enzyme release functions in the sperm head, and a complementary mechanism prevents polyspermic fertilization (Figure 2). Through this systematic review of the microscopic mechanism underlying
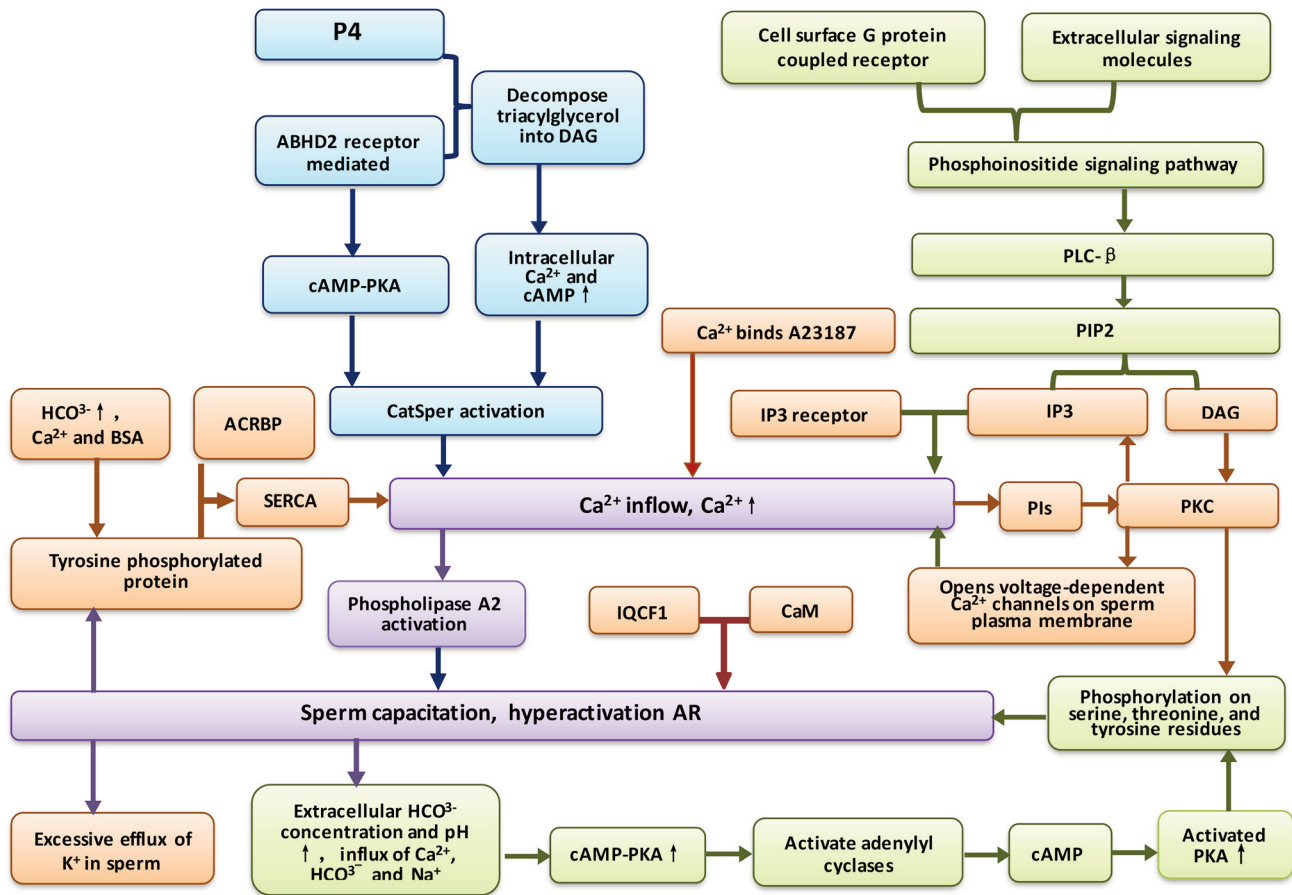
Figure 2. *Fine tuning of the fertilization process: Sperm capacitation. Tyinine phosphate protein and ACRBP can initiate AR through the SERCA pump regulating Ca^{2+} concentration in the cell. The combination of IQCF1 and CaM is closely related to the level of tyrosine phosphorylation and AR. When obtaining capacity, the pH of the female reproductive tract is elevated, and K^+ flows in the sperm, while the extracellular Ca^{2+}, HCO^{3-} and Na^+ induce several cAMP-PKA signal pathways. These pathways activate PKA, so that some sperm proteins are phosphorylated on serine, threonine, especially tyrosine residues causing sperm hyperactivation and AR. HCO^{3-}, Ca^{2+}, and BSA can cause tyrosine phosphorylation mediating sperm capacitation. However, AR can increase the internal Ca^{2+} of the sperm, the pH is elevated, and Ca^{2+} influx activates the PLC-β. After being stimulated by G-protein, the phosphatidylinositols (PIs) on the membrane are hydrolyzed to produce DAG and phosphoinositide. DAG activates PLA2, which leads to the fusion of the outer acrosomal and plasma membrane. When IP3R is stimulated by intracellular IP3, stored Ca^{2+} is released and directly or indirectly causes influx of exogenous Ca^{2+}. Acrosome Ca^{2+} release triggers tyrosine phosphorylation leading to exocytosis. Ca^{2+} induces activation of the PPI signaling pathway and PKC, which in turn activate the opening of Ca^{2+} channels on the sperm membrane to promote the Ca^{2+} influx and AR. Progesterone regulates sperm fertilization via the ABHD2-mediated cAMP-PKA signaling pathway and activates the CatSper of the sperm via ABHD2 to induce Ca^{2+} influx without activating nuclear receptors. After binding with progesterone, ABHD2 is activated to decompose triacylglycerol fats to produce DAG and activate CatSper. Ca^{2+} influx and increased cAMP activate sperm capacitation, hyperactivation, chemotaxis, and AR. Activated ACs increase intracellular cAMP that activates PKA to phosphorylate proteins on serine, threonine and tyrosine residues, thereby regulating sperm capacitation, hyperactivation, chemotaxis, and AR. A23187 binds to Ca^{2+}, induces Ca^{2+} influx, and initiates the fusion of sperm plasma membrane and acrosomal outer membrane leading to AR. See the text for details. ABHD2: Abhydrolase domain-containing protein 2; AC: adenylyl cyclase; ACRBP: acrosin binding protein; AR: acrosome reaction; BSA: bovine serum albumin; CaM: calmodulin; cAMP: 3'5'-cyclic adenosine monophosphate; CatSper: cation channel of sperm; DAG: diacylglycerol; IP3: inositol 1,4,5-trisphosphate; IQCF1: IQ motif containing F1; PIs: phosphatidylinositols; P4: progesterone; PIP2: phosphatidylinositol 4,5-bisphosphate; PKA: protein kinase A; PKC: protein kinase C; PLC-β: phospholipase C-β; SERCA: sarco/endoplasmic reticulum Ca^{2+}-ATPase.*

the fertilization process, we clarified the precise molecular processes regulating fertilization, providing direction for future research and the development of novel treatments. However, individual differences may cause male infertility, and prospective treatments based on this research have not yet been investigated in terms of those individual differences. In future research, the mechanism of fertilization should be thoroughly investigated in combination with individualized treatment protocols, thereby providing better solutions for male infertility and theoretical support for its prevention and treatment.

Conflicts of Interest

The Authors declare that they have no competing interests in relation to this study.

Authors' Contributions

ZH and MX were involved in the design of the work, literature search, analysis and interpretation of data, and drafted the article. QQL, JD, and XL supervised the study and were involved in the critical revision of the article. All Authors have read and approved the final manuscript for publication.

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