CatSper channel, sperm function and male fertility

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Abstract A number of physiological events, such as sperm hyperactivation, chemotaxis towards the egg, capacitation and acrosome reaction, are triggered by activation of sperm ion channels in response to a diverse range of chemical cues. Cation channel of sperm (CatSper), a sperm-specific ion channel, is unique in orchestrating the events for fertilization, and seems to be exclusively evolved for sperm function and male fertility. CatSper acts as a polymodal, chemosensory calcium channel and plays a vital role in the regulation of sperm hyperactivation. CatSper knockout models and application of patch clamp recordings have shown that it is indispensable for male fertility, and mutations and deletions in CatSper gene(s) may lead to infertility. In fact, mutations in CatSper1 and 2 have been identified in infertile individuals; however, CatSper3 and 4 have not been explored. Restricted localization and expression of CatSper in sperm offer an added advantage to developing gamete-based safe non-hormonal contraceptives. This review concisely covers identification, structure, function, and mechanism of action of CatSper channels. The functional importance of this complex ion channel in sperm motility and male fertility is highlighted for further research on male fertility, infertility, and contraception.

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KEYWORDS: CatSper channel, male fertility, male infertility, sperm function, spermatogenesis

Introduction

Cation channel of sperm (CatSper) is a sperm-specific, weakly voltage-dependent, Ca$^{2+}$ selective, pH-sensitive ion channel that controls the entry of positively charged calcium ions into sperm cells, which is essential for sperm hyperactivation and male fertility (Kirichok et al., 2006; Lishko et al., 2011; Shukla et al., 2012). It is noticeable that, similar to other voltage-gated calcium channels, CatSper can conduct monovalent cations such as sodium (Na$^+$) and caesium (Cs$^+$) in the absence of divalent cations; however, the affinity of CatSper to divalent cations (e.g. calcium ion [Ca$^{2+}$]) is much higher. Moreover, under normal physiological conditions, sperm membrane...
potential and intracellular pH are at values such that CatSper channels remain active at a minimal level, which are potentially activated in the presence of agonists or physiological stimuli (Kirichok et al., 2006). Although several prototypical voltage-gated calcium channels are thought to be present in spermatozoa, probably not all of them are essentially required for motility. A number of voltage-gated calcium ion channels have been identified in testis, spermatocytes, and spermatozoa (Babcock and Pfeiffer, 1987; Fragale et al., 2000; Lievano et al., 1996; Serrano et al., 1999; Westenbroek and Babcock, 1999). Interestingly, some of the ion channel proteins are equally expressed in other tissues, such as brain, making it difficult to distinguish the ion channels with a unique functional significance for sperm physiology (Benoff et al., 2007). Ren and Xia (2010) have, therefore, suggested a few hallmark features for identifying ion channels that are important for sperm physiology; the protein must be detectable in sperm, and must be confirmed with knockout sperm as a negative control for antibody specificity; the whole sperm current is detectable by patch-clamp recordings; the application of highly selective sperm ion channel blockers impairs the sperm function by affecting its natural physiological activities; mutations in the gene or targeted disruption of the gene encoding the protein lead to defects in sperm physiology and male fertility.

A protein that does not follow the above mentioned criteria could still be of physiological significance to spermatozoa in vivo. CatSper satisfies most of the criteria proposed above, making it a channel of physiological significance to spermatozoa (Ren and Xia, 2010). An investigation into CatSper orthologs revealed its presence in all mammals examined so far (e.g. human, chimpanzee, dog and rat); however, the identity between CatSper orthologs may differ between species (Cai and Clapham, 2008). In addition to mammals, CatSper genes are also found in the genomes of reptiles, tunicates, echinoderms and cnidarians, but not in the genomes of birds, amphibians, insects, fishes, flies, worms and plants. Evolutionary biologists suggest that, during the process of natural selection, the species with thicker oocyte (e.g. mouse and human) maintained CatSper functionality in its male counterparts, whereas the species with thinner oocyte lost CatSpers, as in the cases of birds and fish (Cai and Clapham, 2008; Navarro et al., 2008).

The CatSper channel is confined to the principal piece of sperm flagellum as shown by localization pattern in mice (Ren et al., 2001) and humans (Cheon et al., 2004) (Figure 1). Influx of Ca\textsuperscript{2+} leads to a rise in sperm intracellular calcium concentration, which facilitates vigorous and faster sperm movements. Moreover, this unique class of Ca\textsuperscript{2+} channel has been shown to be essential for hyperactivated sperm motility, sperm detachment from the epithelium of the female reproductive tract, egg coat penetration and fertility (Ho et al., 2009; Qi et al., 2007). Hyperactivated motility, which is characterized by asymmetrical high amplitude and lower frequency flagellar beating (Figure 2), is essential for releasing sperm from the isthmic reservoir to reach the site of fertilization that is followed by penetration through the egg’s zona pellucida, the extracellular coat that surrounds the egg (Demott and Suarez, 1992; Stauss et al., 1995; Suarez, 2008). The CatSper channel, in association with other ion channels and pumps, facilitates the entry of calcium necessary for rapid changes in sperm motility, allowing the spermatozoa to navigate through the hurdles of the female reproductive tract in order to locate the egg for fertilization (Barratt and Publicover, 2012). The spermatozoon is likely to receive multiple signals in the female reproductive tract, which provide cues and information to the spermatozoon for gaining hyperactivated motility and fertilizing the egg. CatSper channel mutations in humans have been associated with infertility (Hildebrand et al., 2010).

**Figure 1** Sperm geometry: a mature spermatozoon consists of head, neck, mid-piece and tail. Head includes acrosome and nucleus whereas tail has principal piece and the end piece.

**Figure 2** Hypermotility: normal sperm display vigorous and asymmetrical flagellar beating termed as hypermotility, which is essential for propagation through viscous oviductal fluid. CatSper null sperm show only basal motility and are unable to propagate to destination and penetrate protective layers of the egg.
Architecture of the Catsper channel complex

CatSper is a heterotetrameric Ca$^{2+}$ channel composed of four separate pore-forming $\alpha$ (alpha) subunits. These are CatSper 1–4 and three additional auxiliary subunits: CatSper $\beta$ (beta), CatSper $\gamma$ (gamma) and CatSper $\delta$ (delta), encoded by at least seven genes (Figure 3). The involvement of several subunits makes the channel complex; however, such a complexity seems to be required for its functional co-ordination, localization to the flagella, and sensitivity to intracellular pH, progesterone, prostaglandins, odorants, and perhaps to some other proteins and cell-signalling molecules and activators (Darzson et al., 2011; Lishko et al., 2012). The first pore-forming CatSper subunit, CatSper 1, was discovered in 2001, and it was found exclusively in spermatozoa and to be essential for male fertility. CatSper1 was detected during a search for sequence homology to the voltage-gated Ca$^{2+}$ selective channels (Ren et al., 2001). The pore-forming alpha subunits of CatSper channel have high homology to Ca$^{2+}$ selective channels, with highest homology with CatSper 1 subunit. A signature sequence, Thr/Ser-x-Glu/Asp-x-Trp in the ion selectivity filter region, is a common feature to both CatSpers and Ca$^{2+}$ selective channels (Lobley et al., 2003; Ren and Xia, 2010). The mouse CatSper1 gene on chromosome 19 encodes a 686 amino acid protein whereas CatSper2 gene, located on chromosome 2, encodes a protein of 588 amino acids (Navarro et al., 2008). CatSper2 was identified as a sperm cell-specific transcript using a signal peptide trapping method (Quill et al., 2001). The other two members of the family, CatSper3 and CatSper4, were identified using an in-silico database mining (Lobley et al., 2003). CatSper3, located on mouse chromosomes 13, encodes a protein of 395 amino acids whereas CatSper 4, mapped on mouse chromosome 4, encodes a 442 amino acid protein.

The four CatSper subunits have relatively low sequence homology in the transmembrane regions, varying from 16–22% (Navarro et al., 2008). Human CatSper has a high degree of sequence identity (55% identity) with its mouse counterpart; 81% identity in the transmembrane domain and 89% in the pore regions (Hildebrand et al., 2010; Ren et al., 2001). Each of the four separate pore-forming CatSper $\alpha$ subunits has six transmembrane segments (S1–S6) having two functionally distinct modules: the voltage sensor domain (S1–S4) and the pore-forming domain (S1-P loop-S6). It is worth noting that fourth transmembrane segment (S4) of CatSper $\alpha$ subunits (CatSper 1–4) contains several positively charged amino acid residues (lysine/arginine). CatSper1 contains six positively charged amino acid (lysine/arginine) residues aligned in the same manner as in strongly voltage-sensitive channels. CatSper2, however, contains four such residues and only two are preserved in CatSper3 and CatSper4 (Ren et al., 2001). CatSper $\beta$ was the first reported auxiliary subunit of CatSper, identified using database search and reverse transcription polymerase chain reaction. CatSper $\beta$ gene is located on chromosome 12 in mice, and is relatively less conserved between humans and mice. CatSper $\beta$ contains two transmembrane segments with two short cytoplasmic domains and a large extracellular domain (Liu et al., 2007). Another auxiliary subunit, CatSper $\gamma$, is encoded by a gene on chromosome 7 in mice, and consists of a single transmembrane segment with a large extracellular domain and a short cytoplasmic tail (Wang et al., 2009). CatSper $\delta$ gene was recently identified by a database

Figure 3 Functioning and regulation of CatSper: Sperm Na$^{+}$/H$^{+}$ exchanger acts via cyclic adenosine monophosphate, which is generated in response to bicarbonate (HCO$_3^-$). CatSper channel is triggered by increase in intracellular pH, which allows calcium ions to enter in the sperm flagellar cytoplasm. Calcium balance inside the flagellum is maintained by Ca$^{2+}$ adenosine triphosphatase pumps, which export intracellular Ca$^{2+}$ ion and import extracellular protons. The resulting acidification is regulated by proton extrusion via Voltage-gated H$^{+}$ channel 1 (Hv1) channel. Sperm specific K$^{+}$ channel (SLO3) maintains flagellar membrane potential.
search and encodes a sub-unit having a single transmembrane segment with a large extracellular domain and a short cytoplasmic tail (Chung et al., 2011). The chromosomal locations and Ensembl gene identifiers of CatSper subunits of mice and humans are listed in Table 1.

CatSper expression and localization

The CatSper channel is exclusively expressed in testis and precisely localized to the principal piece of the sperm tail. Indirect immunogold electron microscopy detects CatSper immunoreactive gold particles in the plasma membrane above the fibrous sheath in the sperm principal piece. The detection of gold particles on the cytoplasmic face of the plasma membrane supports the predicted intracellular localization of the N-terminus of CatSper (Ren et al., 2001). Gene expression profiling studies on mice and human testis and in situ hybridization studies of CatSper subunits suggest that CatSpers are differentially transcribed at the time of spermatogenesis. CatSper1 (Ren et al., 2001), CatSper3, and CatSper4 transcripts (Jin et al., 2005; Qi et al., 2007; Schultz et al., 2003) are restricted to late-stage germ line cells (spermatids), whereas CatSper2 is transcribed in the early stages of spermatogenesis (pachytene spermatocytes) (Quill et al., 2001; Schultz et al., 2003). CatSper β (Liu et al., 2007), CatSper γ (Wang et al., 2009), and CatSper δ (Chung et al., 2011) are expressed in spermatocyte and spermatids of testes and are strictly localized to the principal piece of spermatozoon. CatSper localization to the principal piece of spermatozoon has been established using immunolocalization experiments (Qi et al., 2007; Quill et al., 2001; Ren et al., 2001). This was confirmed by detection of CatSper current (ICatSper) in the principal piece using patch-clamp recording, as ICatSper could be recorded from the principal piece and mid-piece fragment and not from the head and midpiece fragment (Kirichok and Lishko, 2011; Kirichok et al., 2006). CatSper gene expression was low (up to 3.5-fold difference) in sub-fertile men characterized by loss of sperm motility compared with those having no motility defects, suggesting a possible correlation between lower CatSper gene expression and defective sperm motility in a proportion of sub-fertile patients (Nikpoor et al., 2004). Nevertheless, CatSper channel-associated proteins, such as voltage-gated proton channel (Hv1) and sperm specific K+ channel (Ksper/Slo3), seem to be strictly localized to the principal piece of spermatozoon (Lishko and Kirichok, 2010; Lishko et al., 2010; Navarro et al., 2007).

Functional significance of CatSper channels

Knockout studies have proven that pore-forming subunits and auxiliary subunits are functionally expressed in co-ordination. Interestingly, all CatSper subunits function in coordination with each other, as CatSper β, CatSper γ, CatSper δ, CatSper2, CatSper3, and CatSper4 are all undetectable on CatSper1 null sperm plasma membranes (Carlson et al., 2005; Chung et al., 2011; Liu et al., 2007; Qi et al., 2007; Wang et al., 2009). Notably, CatSper δ null mice are infertile, and the amount of CatSper1 in spermatozoa is remarkably reduced, indicating the essentiality of CatSperδ subunit for formation of a functional CatSper channel (Chung et al., 2011). Targeted disruption of CatSper 1–4 genes in mice leads to complete loss of ICatSper and identical phenotype of male infertility indicating that all pore forming CatSper α subunits are essential for a functional channel (Carlson et al., 2005; Jin et al., 2007; Qi et al., 2007). CatSper δ knockout results in complete loss of CatSper currents and hence male infertility (Chung et al., 2011). Patch clamp recordings of mouse spermatozoa revealed the origin of ICatSper from the principal piece that corresponds to antibody localization of the CatSper protein (Kirichok et al., 2006; Qi et al., 2007; Quill et al., 2001; Ren et al., 2001). ICatSper increases intracellular Ca2+ concentrations [Ca2+]i that eventually leads to hyperactivated motility.

Knockout studies have shown that loss of either of the four pore-forming CatSper sub-units or CatSper δ results in loss of hyperactivated motility caused by the lack of ICatSper mediated Ca2+ entry in the intracellular space of flagellum. ICatSper is weakly voltage dependent (slope factor k = 30) compared with strongly voltage activated channels (k = 4) and has species-specific differences as human ICatSper is slightly steeper than mouse (slope factor k = 20 in human versus k = 30 in mouse) (Kirichok et al., 2006; Lishko et al., 2011). It is speculated that positively charged residues of CatSper α subunits are directly related to voltage sensitivity, and as other pore-forming CatSper α subunits except CatSper1 have a lower number of such residues, the overall voltage sensitivity of the channel is weak (Navarro et al., 2008; Ren et al., 2001). Furthermore, the V1/2 (the voltage at which half of channels are active) of human CatSper is more positive (+85 mV) than mouse (+11 mV) at the same intracellular pH (7.5) (Kirichok et al., 2006; Lishko et al., 2011). The CatSper channel is regulated by changes in intracellular pH, such that the ICatSper is increased with an increase in intracellular pH. Histidine abundance in the N-terminal domain of CatSper1 that varies in

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Table 1 Chromosomal location and ensemble gene identifiers for CatSper subunits.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Chromosome (human)</th>
<th>Ensemble identifier</th>
<th>Chromosome (mouse)</th>
<th>Ensemble identifier</th>
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<tr>
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<td>19A</td>
<td>ENSMUSG00000038498</td>
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<td>2E5</td>
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<td>ENSG00000152705</td>
<td>13B1</td>
<td>ENSMUSG00000021499</td>
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<tr>
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<td>4D3</td>
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<tr>
<td>CatSper γ</td>
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<tr>
<td>CatSper δ</td>
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<td>ENSMUSG00000040828</td>
</tr>
</tbody>
</table>
length and sequence among species (51 His out of 250-amino acid residues in the mouse) is suggested to be responsible for pH sensitivity (Kirichok et al., 2006; Ren et al., 2001). An elevation in intracellular pH is the prerequisite for sperm hyperactivation. Marquez and Suarez (2007) showed that extracellular application of cell permeant NH4Cl evokes elevation of intracellular pH, which stimulates Ca2+ influx and induces sperm hyperactivation in bovine (Marquez and Suarez, 2007), but NH4Cl has negligible effect on human sperm hyperactivation (Alasmari et al., 2013).

CatSper regulation and signalling

CatSper channel involves true polymodal regulation in association with its direct and indirect activators. Consistent with a role of Ca2+ entry in the fertilization process, several of the chemical cues associated with fertilization, such as follicular fluid, progesterone, and prostaglandins, have been shown to induce Ca2+ influx, presumably through Ca2+-permeable ion channels (Brenker et al., 2012). The role of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) in induction of Ca2+ influx through CatSper is controversial as there is no clear evidence suggesting a role of cyclic nucleotide in elevation of [Ca2+]; however, earlier studies using cell permeable cyclic nucleotide analogues (e.g. 8-Br-cAMP and 8-Br-cGMP) propose an indirect role of cAMP/cGMP (Kobori et al., 2000). The use of 8-Br-cNMP may be physiologically irrelevant, as this chemical analogue does not really mimic the native environment of the oviduct. Other factors that affect sperm function by changing [Ca2+]i include chemokines, neurotransmitters and odorants. Progesterone and prostaglandins in humans stimulate Ca2+ influx through direct activation of CatSper (Brenker et al., 2012; Lishko et al., 2011). Progesterone is released by the ovaries, and the cumulus cells surrounding the egg initiate a high Ca2+ influx into human spermatozoon that eventually results in the initiation of sperm hyperactivation and subsequently the acrosome reaction to reach the prerequisite for successful fertilization. (Blackmore et al., 1990). The sensitivity of spermatozoa to progesterone depends on their developmental stage as Smith et al. (2013) have found that sensitivity to progesterone increases with the course of sperm development and maturation, such that maximum sensitivity is seen in ejaculated spermatozoa (Smith et al., 2013). We speculate that variation in sensitivity may act as a filter to achieving quality control in the female reproductive tract so that only fully mature and good quality spermatozoa fertilize the egg. Prostaglandins are also thought to evoke Ca2+ influx and ligands, particularly odorants, directly activate CatSper without involving G-protein-coupled receptors and cyclic adenosine monophosphate (Brenker et al., 2012).

The regulation of CatSper channel may differ significantly between species owing to differences in orthologs of CatSper subunits. CatSper channel of murine spermatozoa is not sensitive to the activators of human CatSper, such as progesterone and prostaglandins (Lishko et al., 2011). Similarly, CatSper is activated by extracellular 8-Br-cAMP in human but not in mouse spermatozoa. It is worth noting that in human spermatozoa, only extracellular application of 8-Br-cAMP, but not intracellular cAMPs directly activates CatSper (Brenker et al., 2012). The above mentioned facts reflect the differences in CatSper channel activation mechanism and evolutionary trends in CatSper genes (Lishko et al., 2012). Recently, Schiffer et al. (2014) have established the adverse role of endocrine disrupting chemicals on human CatSper channel. Premature activation of CatSper channel by endocrine disrupting chemicals desensitizes spermatozoa to physiological CatSper ligands, such as progesterone and prostaglandins, and may, therefore, impair the precisely co-ordinated sequence of fertilization (Schiffer et al., 2014).

In addition to the above mentioned activators, other ion channels, such as Hv1, K+ channel of spermatozoa (Ksper/SLO) and ion pumps, such as plasma membrane Ca2+-adenosine triphosphatase (ATPase), Na+/Ca2+ exchanger, and sperm Na+/H+ exchanger have been proposed to work in close association with the CatSper complex (Figure 3). Intracellular Ca2+ and intracellular hydrogen ion [H+;i], are well known key factors with antagonistic effects on motility and fertilizing ability of sperm, and the contribution of these signalling molecules is mainly determined by Ca2+ and hydrogen ion channels (Darsonz et al., 2006). Calcium stores of acrosome vesicle and the redundant nuclear envelope, however, may also contribute to sperm intracellular Ca2+ signalling (Costello et al., 2009; Ho and Suarez, 2001; Suarez, 2008). In spermatozoa, high resting [H+]i and low resting intracellular Ca2+ suppress the activity of the sperm cell whereas activation of spermatozoa requires reduction of [H+]i and elevation of [Ca2+]i; which are maintained by [H+]i and [Ca2+]i pumps, respectively. Particularly, spermatozoa have two flagellar Ca2+ transport proteins that balance the concentration of Ca2+ in the sperm flagellum; Ca2+ ATPase 4 that pumps Ca2+ out of the spermatozoon and CatSper channel that allows the entry of extracellular Ca2+ inside the spermatozoon (Okunade et al., 2004; Schuh et al., 2004). Sodium calcium exchanger also plays an essential role in Ca2+ extrusion from sperm intracellular environment. The intracellular [Ca2+]i overload is overcome by exporting an intracellular Ca2+ ion and importing three Na+ ions. Interestingly, the activity of this exchanger is highly pronounced in epididymal sperm compared with ejaculated sperm (Rufo et al., 1984). This Na+/Ca2+ exchanger is equally efficient in both ways, and its bimodal operation of ion import and export helps in maintaining Ca2+ homeostasis (Krasznai et al., 2006; Wemmuth et al., 2003).

The regulation of sperm intracellular pH is maintained by H+ conductance in sperm plasma membrane, which involves voltage-gated H+ channel, Hv1 (Florman et al., 2010). In human spermatozoa, Hv1 is localized within the principal piece whereas there is no clear report of hv1 in mice (Lishko and Kirichok, 2010). After experimental confirmation, Hv1 is considered to be responsible for intracellular alkalization that involves H+ extrusion from spermatozoa. Moreover, localization of Hv1 in the principal piece of spermatozoon revealed its importance in the regulation of pH dependent CatSper channel. In contrast to human, Hv1 current was not detected in mouse spermatozoa, and Hv1 knockout mice were reported to be fertile. The exact role of Hv1 in maintaining intracellular alkalization and species-specific functional differences are subject to further investigations (Lishko and Kirichok, 2010; Lishko et al., 2010, 2012). Ksper/SLO3 is a pH sensitive channel made up of seven transmembrane helices, which hyperpolarizes mouse spermatozoa during capacitation. The process of hyperpolarization facilitates Ca2+ entry through CatSper channels by some as yet unidentified
mechanism. Male mice deficient in SLO3 have significantly impaired sperm motility, which leads to infertility (Navarro et al., 2007; Santi et al., 2010; Schreiber et al., 1998; Visconti et al., 2011). Sperm Na⁺/H⁺ exchanger acts via soluble adenylyl cyclase (sAC) that generates cAMP in spermatozoa in response to physiological stimuli, such as bicarbonate (HCO₃⁻) (Carlson et al., 2007; Wang et al., 2007). sAC-dependent elevation of intracellular cAMP facilitates CatSper dependent Ca²⁺ entry via PKA-dependent phosphorylation (Visconti et al., 2011). It was also confirmed by the absence of full length sAC protein in sACE(-/-) and best illustrated by the sterile phenotype in mice deficient in sAC (Esposito et al., 2004; Xie et al., 2006). Other transporters that have been suggested to participate in regulation of sperm intracellular pH are Na⁺/Cl⁻/HCO₃⁻/Cl⁻/HCO₃⁻, and cystic fibrosis transmembrane conductance regulator (Chavez et al., 2012; Zeng et al., 1996).

Precisely, CatSper channel involves true polymodal regulation in association with other channels. Ca²⁺ influx through CatSper channel increases flagellar bending in a Ca²⁺ dependent manner and might involve binding to calmodulin (a calcium binding protein), activation of calmodulin kinase, and phosphorylation of radial spoke proteins of the sperm axoneme. Low intracellular adenosine triphosphate level in CatSper 1 null spermatozoa reflects its importance in flagellar glycolysis via CatSper specific Ca²⁺ entry. Thus, Ca²⁺ via CatSper alone or in combination with intracellular Ca²⁺ stores is required for sustained ATP production to maintain extended motility and hyperactivation of spermatozoa (Xia et al., 2007). It is worth noting that spermatozoa from CatSper1 and CatSper2-deficient mice can be artificially hyperactivated by adding thimerosal (an organomercury compound) that helps to release calcium from intracellular stores (Marquez et al., 2007) or by procaine (an aminobenzoate compound), which probably induces CatSper-independent Ca²⁺ influx by increasing the permeability of the plasma membrane to calcium (Carlson et al., 2005; McPartlin et al., 2009).

CatSper channel seems to be a polymodal Ca²⁺ signalling node that can integrate the actions of diverse physiological stimuli to regulate multiple physiological activities in sperm. Some of the key stimuli involved in sperm dynamics have been shown to induce CatSper dependent increase in [Ca²⁺]i, which include alkaline depolarization, zona pellucida glycoproteins, and bovine serum albumin (BSA) (Ren and Xia, 2010). The contact between a spermatozoon and egg coat (zona pellucida) triggers Ca²⁺ entry into sperm, as suggested by incubation of spermatozoa with solubilized zona pellucida glycoproteins that stimulates an extracellular Ca²⁺-dependent increase in [Ca²⁺]i in sperm. Zona pellucida induced CatSper channel mediated elevation in Ca²⁺ involves an early rapid phase that occurs in milliseconds to seconds and a sustained phase that extends over several minutes (Ren and Xia, 2010; Ren and Xia, 2009b). ZP1, ZP2, and ZP3 are the proteins that constitute the zona pellucida matrix of egg in mouse (Florman et al., 2008; Wassarman and Litscher, 2009) whereas ZP1, ZP2, ZP3, and ZP4 constitute the same in humans (Gupta et al., 2009). Experiments using gel-purified zona pellucidas indicate that ZP3 exclusively acts as a Ca²⁺ influx-inducing protein in bovine and mouse sperm (Arnault et al., 1996) whereas ZP1 and ZP4 contribute to the same in human (Gupta et al., 2009). How contact between a spermatozoon and the zona pellucida triggers rapid increases in [Ca²⁺]i has been an active area of research for reproductive biologists. Since zona pellicuda-induced increase in [Ca²⁺]i can be blocked by G-protein coupled receptor inhibitors, such as pertussis toxin and phospholipase inhibitor neomycin, sensing of the zona pellucidas by sperm is hypothesized to be mediated by G-protein coupled receptors (Florman et al., 2008). The exact mechanisms and signalling cascade involved in transducing zona pellucida induction to the opening of CatSper channels are currently unknown and subject to further investigations. For several mammalian species, BSA is a well known factor for successful in-vitro sperm capacitation (Baldi et al., 2000). Similar to zona pellucidas, acute application of BSA induces [Ca²⁺]i rise, but it is only a rapid phase of activation that lasts up to 20 s. An increase in [Ca²⁺]i induced by BSA starts in the sperm flagellum and propagates to the head. Albumin also causes CatSper dependent Ca²⁺ influx into mouse spermatozoa, perhaps by modulating CatSper gating. It is thought to work by cholesterol removal that changes lipid composition of sperm plasma membrane, although experiments using cholesterol removal inhibitors did not confirm this (Xia and Ren, 2009a).

Furthermore, all CatSper-dependent [Ca²⁺]i increase triggered by zona pellucidas, BSA, cAMP, and other activators starts in the principal piece. These [Ca²⁺]i changes, however, propagate from principal piece to mid-piece and head within a few seconds (Xia et al., 2007). Similarly, tail-to-head Ca²⁺ transmission has been observed in human sperm stimulated with odorant (Spehr et al., 2004), and in sea urchin sperm induced by egg peptides, such as speract (Wood et al., 2003). All of the physiological stimuli that lead to CatSper-dependent Ca²⁺ influxes are associated with motility change and acrosome reaction. Since CatSper knockout sperm do not show hyperactivated motility, it is hypothesized that CatSper-initiated increase in [Ca²⁺]i in the principal piece is required for hypermotility. Mid-piece of sperm is a hub of mitochondria and an increase in [Ca²⁺]i has the potential to increase production of adenosine triphosphate via Ca²⁺-sensitive dehydrogenases and nicotinamide adenine dinucleotide production. Adenosine triphosphate deficiency in CatSper knockout sperm supports the essentiality of CatSper in sperm dynamics. The rise in intracellular Ca²⁺ in sperm head is usually associated with the acrosome reaction (Xia and Ren, 2009a; Xia et al., 2007); however, the overall physiological reaction induced by zona pellucida glycoproteins, alkaline depolarization, and cyclic nucleotides in the CatSper knockout sperm are almost similar to those of its wild type counterpart, although slight differences in kinetics are obvious.

Specific roles of CatSpers

CatSper is required for sperm hypermotility

Disruption of CatSper gene results in markedly reduced specific motility parameters. A simple microscopic examination to measure the percent motility may be able to identify the difference between CatSper+/− and CatSper−/− sperm. A detailed motility analysis of live sperm, preferably using computer-assisted automated sperm analyzer, however, may reveal significant differences between mutant and wild-type sperm. Computer-assisted sperm analysis shows that specific motility parameters, such as path velocity, progressive velocity, and track speed, are significantly impaired in mutant
sperm (Ren et al., 2001). Although the original description of CatSper1 null mice found a decrease in sperm swimming speeds, (Ren et al., 2001), it was later reported that the initial motility of CatSper null sperm was normal, and the most important defect of CatSper null mice sperm was the lack of hyperactivated motility, which is characterized by an asymmetric and vibrant beating of sperm tail (Carlson et al., 2003). This form of motility generates a more powerful force required for sperm capacitation and successful fertilization (Suarez, 2008). A careful comparison of flagellar beating activities of sperm in wild type and CatSper1 null spermatozoa show that mutant spermatozoa are unable to display hyperactivated motility and their flagellar beating remains symmetric with low curvature and small amplitude even in capacitating medium (Carlson et al., 2003) (Figure 2). Studies have reported that CatSper2 null mouse sperm are less progressively motile than wild type mouse sperm in a highly viscous medium (Quill et al., 2003). Hyperactivation is essential for sperm migration in the oviduct, especially to escape from the lower isthmus, a sperm reservoir in the female reproductive tract. However, in-vivo motility behaviour has been hard to evaluate. As CatSper knockout sperm maintain only basal motility, they represent a model to test the in-vivo mechanism of hyperactivated motility. Furthermore, the mutant sperm are not able to move beyond the oviductal sperm reservoir, most likely because of insufficient mechanical force to elude the adherent epithelial cells in the oviduct or, alternatively, because chemotaxis is lacking (Ho et al., 2009). In the absence of hyperactivated motility, sperm have clear defects in physiological roles, fail to pass through the highly viscous fluid in the oviduct, and penetrate the protective barriers on the egg's surface, the cumulus oophorus and zona pellucida (Ren and Xia, 2010; Suarez and Pacey, 2006).

CatSper is required for egg penetration

To assess the fertilizing ability of CatSper mutant (CatSper−/−) sperm, IVF assay were performed. Incubation of superovulated wild-type female mature eggs with capacitated wild-type or mutant sperm for 24 h showed fertilization of 81% eggs by the wild type sperm, but of no egg by the mutant. Some CatSper−/− sperm adhere to the eggs but are incompetent to penetrate, most probably because of lack of hyperactivated motility (Figure 4). At the time of natural fertilization, sperm penetrate the cumulus oophorus and zona pellucida to fuse with the egg's plasma membrane. Interestingly, incubation of wild-type and mutant sperm with eggs whose outer layers had been enzymatically removed (zona pellucida free eggs) showed penetration and fertilization in both the cases. Therefore, CatSper facilitates penetration through the egg’s outer layers, and perhaps plays no role in egg activation and sperm-egg fusion (Quill et al., 2003; Ren et al., 2001).

CatSper and male infertility

Consistent with restricted expression of CatSper in spermatozoa, defects in the channel cause disturbances in sperm physiology rather than spermatogenesis or spermiogenesis. The phenotypic similarity in each of the four knockouts of CatSper alpha subunits (CatSper1-4) in mouse models and the physical interaction among the proteins suggest that the four CatSper proteins form a heteromeric channel and disruption of any of them may result in channel dysfunction leading to male infertility (Qi et al., 2007). This makes genes encoding CatSper subunits strong candidates for mutation screening in infertile human individuals. Accordingly, CatSper1 and CatSper2 mutations in infertile humans have been found to be correlated with asthenoteratozoospermia (Avenarius et al., 2009; Avidan et al., 2003); however, CatSper3 and CatSper4 mutations in infertile individuals have not been reported to date. Similarly, mutations in CatSper β, CatSper γ (Ren and Xia, 2010) and CatSper δ subunits have not been reported; however, given their importance in the assembly of the complex, the genes encoding these subunits are equally

**Figure 4** Functional significance of CatSper: The protective barriers offer a challenge to sperm. CatSper helps in penetration of zona pellucida layer such that CatSper null sperm are unable to penetrate this layer, failing to fertilize the egg.
good candidates for screening in infertile patients. Loss of function in any of the seven known CatSper subunits may eventually lead to male infertility.

Knock-out studies show that the weight of the body and testes of mutant mice are not different from their wild-type counterparts. Sperm counts from mutant and wild-type caudal epididymides are also not significantly different, and the mutant spermatooza manifest cytologically indistinguishable features. The absence of spermatogenesis defects in CatSperα−/− mice is supported by the lack of morphological differences between the wild-type and mutant mouse testes (Chung et al., 2011; Hildebrand et al., 2010; Liu et al., 2007; Qi et al., 2007; Wang et al., 2009). A large number of infertile individuals do not present any detectable sperm abnormality upon microscopic examination. At the same time, there is no detectable abnormality in their female partners. Loss of hyperactivated motility could be one of the several possible factors behind loss of fertility in such individuals. Therefore, normozoospermic infertile individuals are suitable candidates for analysis of CatSper mutations in human infertility.

CatSper as a target for contraception

On the basis of involvement of CatSper channel in physiological events essential to sperm function, CatSper genes seem to be exclusively evolved for sperm function and male fertility. Human CatSper genes are potential targets for screening in male infertility and perhaps would explain some cases of idiopathic infertility. This makes human CatSper channel an ideal target for contraception as well (Choi et al., 2006; Gorside et al., 2013). Considering their unique structure, restricted expression patterns, and physiological function, drugs specific to CatSpers and/or the auxiliary subunits have the potential to treat motility-related infertility or serve as contraceptives. CatSper blockers, such as HC-056456, prevent hyperactivated motility producing results similar to CatSper null sperm. These CatSper blockers open a way to the development of a new class of contraceptives targeting channel activity selectively (Carlson et al., 2009). An idea of immune contraception was also developed when action of anti CatSper1 immunoglobulin G was evaluated on spermatooza, which significantly reduced the percentage of progressively motile cells and rate of fertilization (Li et al., 2009). As CatSper−/− sperm cannot fertilize eggs, a specific blocker of CatSper might be effective when taken by either men or women (Hildebrand et al., 2010). The restricted localization of CatSper to testicular, epididymal and ejaculated spermatooza suggests that a specific blocker should not affect other tissues, and hence side-effects should be minimum or non-existent (Smith et al., 2013). Normal development and behaviour (including sexual) of the mutant mice supports such a prediction. Finally, as the channel represents a new structure, it may be an excellent target for channel agonists or antagonists. The possibility of exploring CatSper as a target for infertility treatment or contraception opens up a new area for drug discovery and development.

Future questions and perspective

Calcium ions play a critical role in signalling in a wide variety of cells, but in contrast to other Ca2+ signalling processes, such as neuronal communication and muscle contraction, little is known at the molecular level concerning Ca2+ signalling in spermatooza. The most recent Ca2+ permeable ion channels with clearly recognized functions in fertilization are the CatSper channels of sperm (Navarro et al., 2008). A number of questions associated with CatSper signalling remain to be answered. One of the major reasons for the unanswered questions is the failure of CatSper expression in heterologous systems. This may be due to participation of more than seven proteins that have already been characterized and explored (i.e. CatSper proteins 1–4, CatSper β, γ and δ) (Darszon et al., 2011). Moreover, the exact cellular environment and structural attributes like the principal piece still need to be identified for the functional reconstitution of CatSper channels, which may lead to establishment of a functional heterologous expression model. Considering direct and indirect stimuli, what other physiological stimuli in addition to BSA, zona pellucida, and cyclic nucleotides affect CatSper channels remains unknown (Ren and Xia, 2010)? Progesterone secreted from the cells of the cumulus oophorus around the egg have also been shown to evoke [Ca2+]i increase in spermatooza, and these stimuli are mediated through CatSper (Lishko et al., 2011; Strunker et al., 2011). Recent findings have established that spermatozoa-associated progesterone receptors are different from the nuclear progesterone receptors (Lishko et al., 2011; Strunker et al., 2011). The advantage of the non-genomic progesterone receptor is its independence on regulation of gene expression especially when spermatooza are transcriptionally silent cells.

Furthermore, the molecular mechanisms of CatSper channel activation by progesterone, prostaglandins, zona pellucida, BSA and alkalinization are poorly understood. For example, if CatSper conductance increases with intracellular alkalinization, what is the pH-sensing mechanism in the CatSper channel? How do the core and the auxiliary subunits interact with each other during channel activation in response to direct and indirect activators? Studies support the fact that histidine-rich domain of CatSper1 acts as a pH probe and intracellular alkalinization activates the CatSper channel (Kirichok and Lishko, 2011; Ren et al., 2001). A subsequent question would be: how then do ZPs induce intracellular alkalinization? In addition, BSA induced increase in [Ca2+]i, when both voltage and pH are clamped, suggests the existence of another activation pathway in addition to intracellular alkalinization.

Furthermore, recent reports suggest that, in spermatooza, Ca2+ from neck, midpiece or acrosomal stores regulate different activities. In response to progesterone, CatSper mediates Ca2+ influx, which is followed by Ca2+ induced Ca2+ release (CICR) that facilitates the mobilization of Ca2+ store at the sperm neck. The release of stored calcium is likely to be responsible for potent hyperactivation of human sperm perhaps by increased incidences of [Ca2+]i oscillations (Alasmari et al., 2013). Moreover, what is the mechanism of Ca2+ oscillations (repetitive transient [Ca2+]i waves) during tail-to-head [Ca2+]i propagation and what is the functional significance of unidirectional Ca2+ propagation are further questions to be addressed? These questions can be answered by further research in the area of sperm Ca2+ signalling and electrophysiology of ion channels (Ren and Xia, 2010). A different behaviour is seen with solubilized zona pellucida, which stimulates generation of inositol triphosphate in mouse...
spermatozoa that is suggested to bind inositol triphosphate receptors of acrosome and mobilize acrosomal Ca\(^{2+}\) store before the acrosome reaction (Florman et al., 2008; O'Toole et al., 2000). Apparently, [Ca\(^{2+}\)]i signals mediated by alkalization and by stored Ca\(^{2+}\) have separate but interlinked signalling mechanisms to regulate different sperm behaviours. The exact mechanism of stored Ca\(^{2+}\) signalling cascade, however, and its interaction with other Ca\(^{2+}\) stores in response to various physiological stimuli needs extended investigation.

Understanding of ion channels and their roles in sperm physiology has become possible as a result of the advancement in direct recordings of sperm ion currents. Patch clamp recordings found spermatozoa to possess a number of physiologically important channels that are essential to sperm function. CatSper is now well known to play an indispensable role in sperm physiology by facilitating sperm hyperactivation before fertilization. CatSper is unique in orchestrating the events for fertilization and appears to be exclusively evolved for sperm function and male fertility. CatSper acts as a polymodal, chemosensory calcium channel and plays a vital role in the regulation of sperm hyperactivation. It seems that Ca\(^{2+}\) influx via CatSper and release of intracellular Ca\(^{2+}\) stores interact in a complex manner to make fertilization possible.

Since CatSper subunits form a functional channel, loss of function of any of the subunits affects channel function and hence fertilizing ability of sperm. It may not be easy to detect defects in CatSper mutation carriers, as normal testicular architecture and sperm production are not affected. Loss of fertility, however, may be seen as a result of fine changes in sperm ion transport and eventual loss of hypermotility. There is enormous scope in undertaking functional genomics experiments aimed at establishing the relationship between CatSper mutations and male infertility. Sperm specific ion channels may be functionally active in one species, but be functionally absent in another (Lishko et al., 2010). Keeping species specific differences in mind, identification and functional characterization of human sperm ion channels is essentially required, which would be helpful in the understanding of fertility and infertility. Insights from knock-out studies have demonstrated that only two sperm specific ion channels, CatSper and KSper, may cause male infertility, but without affecting normal sperm production. This makes these genes important candidates for screening in infertile men. However, studies on humans, such as mutation analysis in infertile individuals, are scarce. Such studies would further help understand the importance of these genes and ion channels in male fertility. Our understanding about the sperm channelopathies may reach to new dimensions by the use of advanced genetic, electrophysiological, and imaging tools.

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