Original Article

Inhibition of CatSper and Hv1 Channels and NOX5 Enzyme Affect Progesterone-Induced Increase of Intracellular Calcium Concentration and ROS Generation in Human Sperm

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What's Known

• CatSper and Hv1 channels are the most important channels in the human sperm that control sperm function. CatSper channel is activated by progesterone and intracellular alkalization.
• NOX5 enzyme activation depends on intracellular calcium concentration

What's New

• CatSper and Hv1 channels play a critical role in increasing intracellular calcium concentration and ROS generation induced by progesterone. There is a functional relationship between channels in increasing calcium concentration.
• ROS generation is augmented by progesterone. NOX5 enzyme, CatSper, and Hv1 channels might be directly/indirectly involved in this process.

Abstract

Background: Normal sperm function depends on appropriate intracellular calcium (Ca_i^{2+}) and reactive oxygen species (ROS) levels. Calcium activates NADPH oxidase-5 (NOX5) that leads to ROS generation. The calcium channel of sperm (CatSper) is activated by progesterone and intracellular alkalization. Herein, the interactive role of CatSper, Hv1 channels, and NOX5 enzyme on Ca_i^{2+} and ROS generation in human sperm is investigated.

Methods: The present laboratory in vitro study was carried out in the School of Medicine, Shiraz University of Medical Sciences (Shiraz, Iran) during 2016. Normal semen samples (n=15) were washed and diluted to 20×10^6 sperm/mL. The diluted samples were divided into 16 groups containing Ham's F-10 (the control group), 2 µM NNC (CatSper inhibitor), 1 mM $ZnCl_2$ (Hv1 inhibitor), 1 µM DPI (NOX5 inhibitor), NNC+Zn, NNC+DPI, and NNC+Zn+DPI. The other 8 groups were the same as the above except that they contained 1 µM progesterone. Cell viability and Ca_i^{2+} were analyzed by flou-3 AM probe and PI staining, respectively, using flow cytometric method. ROS generation was assessed by chemiluminescence method. Statistical analysis was performed using the one-way ANOVA followed by Tukey's test. P values <0.05 were considered statistically significant.

Results: Progesterone increased Ca_i^{2+} and ROS generation. The addition of NNC, Zn, or NNC+Zn significantly decreased Ca_i^{2+} in the control and progesterone containing groups. Progesterone-induced ROS generation was decreased significantly in all groups containing NNC, Zn, or DPI and reached to the control level when DPI was added to NNC or Zn.

Conclusion: There is a functional relationship between CatSper and Hv1 channels in increasing Ca_i^{2+}. The activity of CatSper and Hv1 channels are required for progesterone-induced ROS generation by NOX5 enzyme.

Introduction

Calcium is involved in nearly every event of sperm capacitation and egg penetration.[1] For years, it was questioned as to which

channel is responsible for calcium entry into human sperm. Eventually, some research studies showed that the main pathway for calcium entry is sperm-specific calcium channel called CatSper, which is the most abundant calcium channel on sperm.[2,3] The CatSper channel is restricted to the principal piece of human sperm tail.[1] It is shown that there is the basal calcium current through CatSper, which is blocked by NNC55-0396 (NNC).[4]

CatSper activation mainly depends on progesterone. The patch-clamp technique showed that nanomolar concentration of progesterone strongly induces calcium current through CatSper in human sperm.[4] This progesterone-induced calcium current was inhibited by CatSper channel inhibitors.[3] It has been shown that CatSper acts as a non-genomic receptor for progesterone.[4] Furthermore, intracellular alkalization is another strong activator of CatSper.[5] In fact, there is a histidine-rich domain at the intracellular NH2 terminus of CatSper, related to alkalization sensitivity of the channel.[1] Intracellular alkalization is due to proton exit through flagellar proton channel of sperm called Hv1 channel.[6] Hv1 channel is the main proton extrusion pathway in human sperm.[5,6] Removal of extracellular zinc (Zn) is one of the physiological activators of Hv1 channel.[6] Interestingly, Hv1 and CatSper channels are co-localized in the sperm flagellum. On the other hand, it is suggested that an increase in intracellular calcium (Ca_i^{2+}) causes reactive oxygen species (ROS) generation in human sperm.[7] Calcium stimulates ROS generation through the mitochondria[8] and also membrane NADPH oxidase (NOX) enzymes.[9] The only NOX enzyme found on human sperm is (NOX5).[9] In fact, NOX5 has calcium-binding sites at the end of its amino domain, and an increase in Ca_i^{2+} activates this enzyme and leads to ROS generation.[10] NOX5 is localized in the principal piece of sperm.[9] Low amounts of ROS are an important physiological factor involved in sperm activation.[11] Studies have shown that O_2^- production is one the first events of capacitation and the involvement of ROS generated in adenylyl cyclase and tyrosine activation is clearly demonstrated.[12] Moreover, it is shown that addition of H_2O_2 at low doses leads to capacitation, while the existence of catalase prevents it.[12] Furthermore, one study showed that ROS generation by NOX5 requires Hv1 activation[9] in a way that Hv1 inhibition by Zn prevents ROS generation through NOX5.

Co-localization of NOX5, Hv1, and CatSper in the principal piece of human sperm and functional characteristics of each of them,

encouraged to investigate the interactive function of these proteins on Ca_i^{2+} and ROS generation. The present study aimed to find the nature of the effect of CatSper and Hv1 inhibition on Ca_i^{2+} in control and progesterone-stimulated condition. Additionally, we aimed to identify the interactive function between CatSper and Hv1 channels on intracellular calcium. Moreover, the role of progesterone in inducing ROS generation by NOX5 and the functional interaction of NOX5, Hv1, and CatSper in this process were investigated. Furthermore, we studied the effects of CatSper, Hv1, and NOX5 inhibition on sperm survival to realize the role of these factors on cell viability in the absence and presence of progesterone.

Materials and Methods

Sample Collection and Sperm Isolation

Normal semen samples (n=15) were obtained from healthy fertile men (20-40 years old) based on WHO (World Health Organization) criteria.[13] The study protocol was approved by the Research Ethical Committee of Shiraz University of Medical Sciences (Shiraz, Iran). The participants were chosen among men who did not have any medical problems, did not use alcohol, drugs, or dietary supplements. A written informed consent was obtained from all individuals. Complete instruction on semen sample collection was provided to the donors. Semen samples were collected after 3-5 days of sexual abstinence. Sperm concentration and motility were evaluated by a sperm quality analyzer (SQA–V, Australia, H18-990) after liquefaction. Semen characteristics of the selected samples is shown in table 1.

Sperms were washed with Ham's F-10 as the sperm medium and motile sperm aspirated after swimming up. Then, sperm medium was added to adjust sperm concentration to 20×10^6 sperm/ml. For ROS generation assessment, the adjusted sample was divided into 16 groups, including the control group (Ham's F-10), 2 µM

Table 1: The characteristics of the selected semen samples

Semen characteristics	Mean±SE (n=15)
Sperm concentration ($\times10^6$/ml)	115.6±6.7
Total sperm count ($\times10^6$)	368.1±33.2
Total motility (%)	61.2±2.3
Semen volume (ml)	4.3±0.2
pH	7.8±0.2
Normal morphology (%)	60.1±3.2
Viability (%)	2.2±56.6
Viscosity	Normal

NNC as CatSper inhibitor (Sigma, N0287), 1 mM ZnCl$_2$ (Zn) as Hv1 inhibitor (Sigma, Z0152), 1 µM DPI (Diphenyleneiodonium chloride) as NOX5 inhibitor (Sigma, D2629), NNC+Zn, NNC+DPI, Zn+DPI, and NNC+Zn+DPI. The other eight groups were the same except that all of them contained 1 µM progesterone (P) (Sigma, p8783). Intracellular calcium was assayed in only 10 groups, including the control group (Ham's F-10), NNC, Zn, DPI, and NNC+Zn in the absence or presence of progesterone.

Evaluation of Intracellular Calcium Levels and Cell Viability by Flow Cytometry

Flou-3 AM probe (Sigma, 73881) was dissolved in DMSO with 1 µM final concentration and added to sperm suspension. Then, it was incubated at 37°C and 5% CO2 for 30 minutes. Afterward, the resulting sample was well mixed and transferred into 10 separate but equal microtubes; each group contained 400 µl. In order to remove additional Flou-3 AM, all the microtubes were centrifuged for 5 minutes. The supernatant was discarded and the obtained sediment was re-suspended to a volume of 500 ml using Ham's F10. Then, all the experimental groups were transferred to the flow cytometry tubes. Inhibitors and progesterone were added in accordance with the above-mentioned procedure.

To evaluate cell viability, one minute before reading the samples by flow cytometer, propidium iodide (PI, Sigma, P4170) was dissolved in PBS at 5 mM final concentration and added to the intended groups. For each sample, 50,000 sperm were evaluated by flow cytometer. The results were analyzed using the FlowJo software.

ROS Measurement

Basal and progesterone-stimulated ROS generations were determined by chemiluminescence measurement. Briefly, 300 µl of washed sperm suspension at the concentration of 20×10^6 sperm/ml was placed in 96-well microplates. Then, freshly prepared luminol probe (Sigma, A8511) was dissolved in DMSO (250 µM final concentration) and added to each sample. Horseradish peroxidase (Sigma, P6782) with 12 U/ml final concentration was added to increase chemiluminescence signal. The relative light unit (RLU) was assessed by multi-mode microplate reader (Synergy HT, Biotech, Germany). Quantitative chemiluminescence average signal was evaluated for each group at 37°C every 10 minutes for 30 minutes.

Statistical Analysis

Data were analyzed using the SPSS 16.0 software package. The results were expressed as mean±SD and the data were normally distributed. The statistical comparisons between the groups were done using one-way ANOVA followed by Tukey post hoc test. P<0.05 were considered statistically significant.

Results

Intracellular Calcium

Pseudocolor figures of the control and progesterone groups are shown in figure 1. The results of Flou-3 and PI staining are presented in table 2 and 3. The mean fluorescent intensity (MFI) in Flou-3 positive cells was calculated by the FlowJo software. Progesterone significantly increased MFI (P=0.009) in comparison with the control group (figure 2). Sperm incubation in the medium containing NNC, Zn, or NNC+Zn significantly decreased MFI (P<0.001) in the control and in the presence of progesterone (figure 2). The addition of DPI to the sperm medium did not change the MFI in the presence

Group (n=15) Sperm status	Control	NNC (2 µM)	Zn (1 Mm)	DPI (1 µM)	NNC+Zn
Q1 (Fluo-3⁻PI⁺) (%)	8.75±0.9	11.97±3.1	4.24±1.01[b] P=0.04	8.13±0.6	30.54±4.7[a,b,c,d] P<0.001
Q2 (Fluo-3⁺PI⁺) (%)	1.03±0.1	15.51±2.8[a] P<0.001	1.15±0.3[b] P<0.001	0.63±0.08[b] P<0.001	12.83±3.2[a,c,d] P<0.001
Q3 (Fluo-3⁺PI⁻) (%)	70.88±3.9	28.07±3.3[a] P<0.001	38.06±6.06[a] P<0.001	74.35±3.8[bc] P<0.001	23.72±2.7[a,c,d] a: P<0.001, c: P=0.01, d: P<0.001
Q4 (Fluo-3⁻PI⁻) (%)	19.29±4.09	44.43±4.7[a] P<0.001	56.52±6.8[a] P<0.001	17.42±3.6[b,c] P<0.001	32.04±5.4[c,d] c: P<0.001, d: P=0.04
Total live	90.18±0.9	72.50±3.1[a] P<0.001	94.59±1.09[b] P<0.001	91.77±0.8[b] P<0.001	55.77±4.2[a,b,c,d] P<0.001

Table 2: The flow cytometric data of sperm incubated in 2 µM NNC, 1 mM Zn, and 1 µM DPI

Q1-Q4 represents the dead low calcium, dead high calcium, live high calcium, and live low calcium sperm, respectively. Results are expressed as means±SD. [a]Significant difference with the control group; [b]Significant difference with the NNC group; [c]Significant difference with the Zn group; [d]Significant difference with the DPI group (P<0.05)
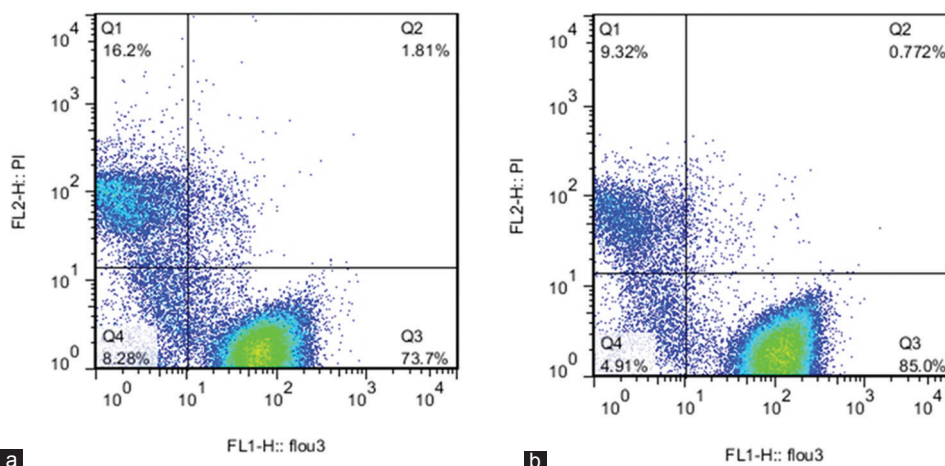
Figure 1: The assessment of intracellular calcium and sperm viability by flow cytometric method in the control (a) and progesterone (b) groups. Q1-Q4 represents the dead low calcium, dead high calcium, live high calcium, and live low calcium sperm, respectively.
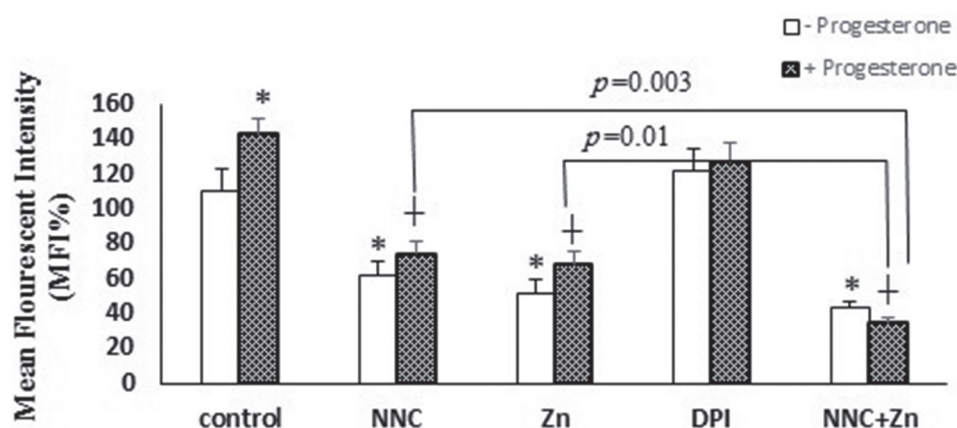

Figure 2: The effects of 1 μM progesterone, 2 μM NNC, 1 mM Zn, and 1 μM DPI on the percent of mean fluorescent intensity (MFI) analyzed by the FlowJo software. P<0.05 were considered statistically significant. *Significant difference with the control group; +Significant difference between the groups containing progesterone.

or absence of progesterone. Hence, the Ca_i^{2+} was not evaluated in groups containing NNC+DPI, Zn+DPI, and NNC+Zn+DPI.

The percentage of sperm viability was reduced significantly (P<0.001) in groups containing NNC and NNC+Zn in the presence or absence of progesterone (table 2 and 3). Sperm incubation in the medium containing DPI or Zn did not change sperm viability in comparison with the control and progesterone groups.

ROS Generation

The 30-minute RLU average is presented in figure 3. Sperm incubation in the medium containing NNC, Zn, and/or DPI did not change the ROS generation in the control condition (absence of progesterone). ROS generation was increased significantly in the progesterone group in comparison with the control (P<0.001). Progesterone-induced ROS generation was decreased significantly in all groups containing NNC, Zn, or DPI as well as in the groups

containing the combination of these inhibitors (figure 3). It should be noted that the ROS decreased in the control level only when DPI was added to NNC and/or Zn groups.

Discussion

The results of the present study showed that the percentage of MFI in Flou-3 AM positive cells increased significantly by progesterone (figure 2). Other reports have shown that progesterone is the most potent physiological factor that induces calcium current in human sperm[4] and thus enhances Ca_i^{2+}. Moreover, it is proven that calcium entry induced by progesterone is through CatSper channel.[3,4] Several studies have shown that mutations or deletions in CatSper results in men infertility.[14-16] Moreover, it is also demonstrated that hyperactivation motility, which is an essential part of capacitation event, is not induced in CatSper knockout sperm.[17,18] It is assumed that CatSper-dependent increase in

calcium in the flagellum is required for motility.[1] All these findings emphasize the importance of calcium in sperm function.

In the present study, the calcium current through CatSper channel was not examined. However, it was shown that inhibition of CatSper by NNC reduces the inducing effect of progesterone on calcium. Likewise, other studies have shown that inhibition of CatSper by NNC abolishes the progesterone-induced calcium current.[3, 4] NNC also decreased Ca_i^{2+} even in the absence of progesterone. Furthermore, NNC decreased the percentage of high calcium live cells (Flou-3⁺ PI⁻) and increased live low-calcium cells (Flou-3⁻ PI⁻) significantly in the presence or absence of progesterone (tables 2 and 3). These findings suggest that CatSper channels are activated even in the absence of progesterone (basal condition). In fact, another report has proved that there is a current through CatSper even in the control condition by applying patch-clamp technique.[4]

As mentioned earlier, sperm intracellular alkalization is another necessary factor for several physiological responses that are required for fertilization, such as calcium influx into sperm, acrosome reaction, and hyperactivation.[2,5] Several reports have shown

Table 3: The flow cytometric data of sperm incubated in 1 μM of progesterone (P) plus 2 μM NNC, 1 mM Zn, and 1 μM DPI or their combination

Group (n=15) Sperm status	P (1 μM)	P+NNC (2 μM)	P+Zn (1 mM)	P+DPI (1 μM)	P+NNC+Zn
Q1 (Fluo-3⁻PI⁺) (%)	7.80±0.6	16.60±4.3 ᵃ P=0.02	7.74±2.9ᵇ P=0.02	8.22±0.5ᵇ P=0.03	25.98±4.08ᵃ,ᵇ,ᶜ,ᵈ a: P<0.001, b: P=0.02, c: P<0.001, d: P<0.001
Q2 (Fluo-3⁺PI⁺) (%)	0.98±0.1	13.48±3.01ᵃ P<0.001	1.32±0.3ᵇ P<0.001	0.95±0.1ᵇ P<0.001	14.25±3.3ᵃ,ᶜ,ᵈ P<0.001
Q3 (Fluo-3⁺PI⁻) (%)	67.32±5.5	22.58±2.8ᵃ P<0.001	44.17±4.05ᵃ,ᵇ P<0.001	70.60±3.6ᵇ,ᶜ P<0.001	31.83±2.6ᵃ,ᶜ,ᵈ a: P<0.001, c: P=0.03, d: P<0.001
Q4 (Fluo-3⁻PI⁻) (%)	23.76±5.5	49.12±6.06ᵃ P<0.001	46.71±4.9ᵃ P<0.001	20.23±3.5ᵇ,ᶜ P<0.001	27.93±3.2ᵇ,ᶜ b: P=0.004, c: P=0.01
Total live	91.09±0.5	71.71±6.01ᵃ P<0.001	90.88±3.1ᵇ P<0.001	90.83±0.5ᵇ P<0.001	59.77±3.7ᵃ,ᵇ,ᶜ,ᵈ a: P<0.001, b: P=0.004, c: P<0.001, d: P<0.001

Q1-Q4 represents the dead low calcium, dead high calcium, live high calcium, and live low calcium sperm, respectively. Results are expressed as means±SD. ᵃSignificant difference with the control group; ᵇSignificant difference with the NNC group; ᶜSignificant difference with the Zn group; ᵈSignificant difference with the DPI group (P<0.05)
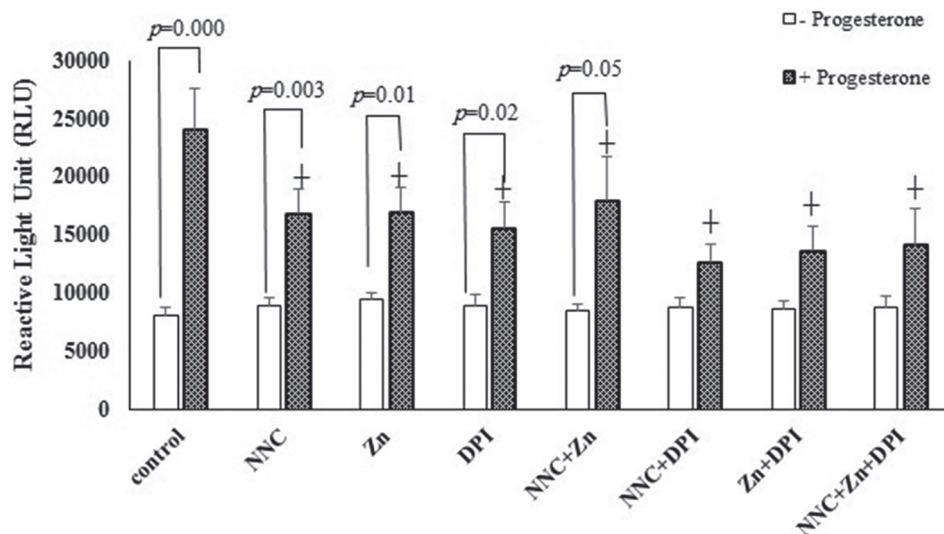

Figure 3: The effects of 1 μM progesterone, 2 μM NNC, 1 mM Zn, and 1 μM DPI on the relative light unit (RLU), which is an indicator of the sperm ROS generation. P<0.05 were considered statistically significant; ‡Significant difference with the progesterone group.

that intracellular alkalization induction leads to calcium concentration enhancement.[2,19,20] After numerous investigations to discover the mechanism responsible for proton extrusion in human sperm, it was shown by patch-clamping that Hv1 proton channel has dominant proton conductance.[2, 6] Hence, Hv1 channel is expected to induce human sperm alkalization.

Sperm Hv1 channel can be activated by the removal of extracellular zinc, a potent blocker of Hv1 channel.[6,21] Zinc in human is highest in seminal plasma (total 2.2 ± 1.1 mM compared with 14 ± 3.0 µM is serum). Seminal zinc inhibits Hv1 channel and sperm activation.[5, 22] However, as sperm travel through the female reproductive tract, zinc bound is released through absorption by the uterine epithelium and chelation by albumin.[5] Hence, at the fertilization site, spermatozoa should be free from zinc inhibition. According to these findings, we hypothesized that intracellular alkalization due to Hv1 activation might lead to calcium entry through CatSper. Our results confirmed the hypothesis and showed that Hv1 inhibition channel results in a significant decrease in Ca_i^{2+}. Besides, we know clearly that calcium entry through CatSper channel is dependent on intracellular alkalization. As one study showed, NH_4Cl evokes Ca^{2+} signals, which is inhibited by NNC.[3] Putting these findings together, it is likely that Hv1 blocking affects intracellular pH and CatSper function.

The MFI in the P+NNC+Zn group was significantly less than the P+NNC or P+Zn containing groups. This effect was not seen in the absence of progesterone (figure 2). This finding could point to the cooperation of CatSper and Hv1 channels when sperm are stimulated with progesterone. Progesterone increased the sperm ROS generation. This effect was reduced by CatSper, Hv1, and NOX5 inhibitors. However, the ROS level generation was not reduced to the control level unless DPI was added to the sperm medium containing NNC or Zn.

It is shown that progesterone induces ROS generation in cultured rat hepatic stellate cells.[23] Another study showed that progesterone has a positive effect on ROS generation too.[24] Similarly, other studies proved the calcium-dependent ROS generation by human spermatozoa.[10, 25-27] Lamirande et al. used calcium ionophore A23187 to induce ROS generation in human sperm.[25] Moreover, it is clearly shown that ROS generation can be blocked by DPI.[10, 26] One study showed a functional connection between Hv1 channel and NOX5 enzyme.[19] This finding showed that superoxide production by NOX5 requires Hv1 channel. Musset et al. showed that the induction of superoxide production in K562/

NOX cells stimulated by ionomycin, PMA, or H_2O_2 was decreased by 1 mM $ZnCl_2$.

According to the above-mentioned findings, CatSper and Hv1 have not a direct role in ROS production, but their activity affects NOX5. Therefore, we recommend a functional relationship between these two channels with NOX5. This concept is not far-fetched since all these molecular complexes are co-localized within the sperm flagellum.[2,6,10]

In the present study, the sperm viability percentage was also evaluated. Blockage of CatSper channel decreased the percentage of cell viability. Regarding the importance of calcium homeostasis in cell survival,[28] it is quite logical that any disturbance in Ca_i^{2+}, especially in a sensitive cell such as sperm, would affect sperm viability. Other reports also showed that the blockage of calcium channel by NNC results in cell death. Ovarian cancer cell line proliferation was inhibited[29] and the apoptosis of leukemia cell line[30] induced by NNC. The mechanism responsible for such effect of NNC on sperm viability is unknown.

The results of the present study would have been confirmed further if other inhibitors and even stimulators of CatSper and Hv1 channels and NOX5 enzyme were used concurrently. Moreover, recording of calcium and proton current through sperm membrane could provide valuable information about the functional interaction between these channels.

Conclusion

Progesterone increased the Ca_i^{2+} of sperm. For the first time, we showed that this effect can be mediated by CatSper in cooperation with Hv1 channels, especially when sperm were stimulated by progesterone. Moreover, it seems that the function of both channels along with NOX5 is necessary for ROS generation induced by progesterone. Further studies are required for a better understanding of the interactive function between CatSper and Hv1 channels and NOX5 enzyme.

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Conflict of Interest: None declared.

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