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Original Article

Inhibition of the CatSper Channel and NOX5 Enzyme Activity Affects the Functions of the Progesterone-Stimulated Human Sperm

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Abstract

Background: Low levels of reactive oxygen species (ROS) and calcium are necessary for sperm function. NADPH oxidase 5 (NOX5) is a membrane enzyme which produces ROS. This enzyme is dependent on calcium for its activity. We investigated the importance of NOX5 and an important calcium channel (CatSper) on sperm function.

Methods: This laboratory *in-vitro* study was done in Shiraz, Iran, 2016. Normal semen samples (n=24) were washed and diluted to 20×10^6 sperm/mL. The diluted samples were divided into 8 groups, containing Ham's F-10 (control group), 2 µM of NNC (CatSper channel inhibitor), 1 µM DPI (NOX5 inhibitor), and NNC+DPI. The other 4 groups were the same as the 1st ones, except that they contained 1 µM of progesterone. Motility assessment was done by VT–Sperm 3.1. Acrosome status was monitored with acrosome-specific FITC-PSA using fluorescent microscopy. Sperm viability was assessed by Eosin Y. Statistical analysis was performed using SPSS 16 software. The comparison between the groups was done using the one-way ANOVA, followed by Tukey. A $P<0.05$ was considered significant.

Results: The percentage of motile sperm, sperm velocity, and viability decreased significantly in the groups containing NNC. DPI reduced sperm progressive motility only in the progesterone-stimulated condition. Progesterone induced acrosome reaction, but this effect was inhibited by NNC and DPI.

Conclusion: CatSper had a prominent role in the motility, acrosome reaction, and viability of the human sperm. The function of NOX5 was important only in the stimulated sperm. We conclude that CatSper has a more prominent role than NOX5 activity. The functional relation between NOX5 and CatSper is not clear but is very probable.

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Keywords ● NOX5 protein ● Human ● CatSper ● Progesterone ● Sperm ● Acrosome reaction

What's Known

- We showed CatSper channel inhibition affects sperm motility, acrosome reaction, and sperm viability in stimulated and unstimulated conditions.
- NOX5 enzyme activity is not important in unstimulated sperm motility, acrosome reaction, and sperm viability. The role of this enzyme is prominent when the sperm is stimulated by progesterone.

What's New

- Progesterone induces calcium entry into the human sperm through the CatSper channel.
- Calcium has a main role in sperm motility and acrosome reaction. Furthermore, calcium is a key activator of the NOX5 enzyme.

Introduction

Low intracellular concentrations of reactive oxygen species (ROS) constitute a physiological factor that affects the activities of the human sperm such as motility, acrosome reaction (AR), capacitation, and fertilization.[1,2]

The exact production sources of ROS by the human sperm are not fully known. However, mitochondria and also membrane NADPH oxidase (NOX) are main candidates as the source of ROS in the human sperm.[3,4]

So far, 5 types of NOX enzymes have been identified in mammals which transfer electrons to oxygen, causing superoxide anion production.[5,6] None of the NOX 1–4 enzymes has been identified in the human sperm. The only known NOX enzyme that is detected in the human sperm is called NOX5.[4,7] In fact, the gene of NOX5 is found on chromosome 15.[3] The expression of the NOX5 enzyme is proven by using an antibody that is specific for the NOX5 enzyme.[4,7]

Among NADPH oxidases, the NOX5 enzyme has unique properties. This enzyme has 4 binding sites for calcium at the end of its amino domain[8] and calcium ion is essential for its activation[5] such that NOX5 activity increases significantly in response to calcium ionophore ionomycin[4] and decreases with the removal of calcium from the outside of the cell and also decreases by diphenyleneiodonium (DPI), a nonspecific inhibitor of the NOX5 enzyme.[4] Moreover, it has been proven that progesterone induces calcium entry into the human sperm.[9] Some recent studies have shown that calcium entry by progesterone is through a sperm-specific calcium channel called "the CatSper channel".[9,10] It is shown that the disruption of the CatSper channel abolishes the progesterone-sensitive current through the CatSper channel and results in men infertility.[11] By doing so, progesterone controls several functions of the human sperm that are associated with an increase in the intracellular calcium such as motility and AR.

Overall, progesterone is one of the most important stimulators of the human sperm. For instance, progesterone induces calcium entry into the human sperm through the CatSper channel and calcium is the main activator of the NOX5 enzyme.

To determine the importance of NOX5 and CatSper activity in sperm function, we investigated the effects of the inhibition of NOX5 and the CatSper channel on motility, AR, and viability in the presence and absence of progesterone.

Materials and Methods

Participants

Human semen samples (n=24) were obtained from fertile men (20–40 years old), who had referred to Shiraz Infertility Centre, Shiraz, Iran, in 2016. The study protocol for this laboratory study was approved by the Research Ethics Committee of Shiraz University of Medical Sciences (Ethical Code: EC–92–6773). A written informed consent was obtained from all the participants in this study. Donors were chosen from among nonsmoking men who did not have any medical problems and had not used any drug, dietary supplements, and alcohol. In terms of fertility, they were totally healthy. Semen was collected by masturbation after 3–5 days of sexual abstinence. Complete instruction as to how to collect the semen sample was provided to the donors. Normal semen samples from healthy men were chosen based on the WHO's criteria.[12]

Semen Samples and Sperm Isolation

Total semen volume, viscosity, pH, appearance, sperm concentration, and motility were assayed after liquefactions with a SQA-V™ sperm quality analyzer (Austria, H18-990). Normal liquefied semen samples were loaded with a sperm medium (Ham's F-10) and centrifuged at room temperature. The pellets were re-suspended in 0.5 mL of the sperm medium and then incubated for 60 minutes at 37°C under 5% CO_2. Motile spermatozoa that progressed from the pellet into the supernatant were collected by aspiration. The number of sperm in suspension was counted using a Neubauer hemocytometer under a light microscope (CX41, Japan). Then according to the number of sperm, Ham's F-10 was added to adjust sperm concentration to 20×10^6 sperm/mL. Subsequently, the adjusted sample was divided into 8 experimental groups, containing Ham's F-10 (control group),1 µM of progesterone (P8783, Sigma Aldrich, Germany), 2 µM of NNC (N0287, Sigma Aldrich, Germany) as CatSper inhibitor, 1 µM of DPI (D2629, Sigma Aldrich, Germany) as NOX5 inhibitor, NNC+DPI, NNC+progesterone, DPI+progesterone, and NNC+DPI+progesterone groups. Thereafter, the samples were incubated at 37 °C and 5% CO_2 for 30 minutes. Finally, sperm motility, AR, and viability were evaluated.

Motility Assessment

Sperm motility is categorized into progressive, nonprogressive, and immotile according to the WHO's criteria.[12] Motility is progressive when spermatozoa move linearly or in a large circle. Nonprogressive motility is all the other patterns of motility except progressive movement such as swimming in small circles. Immotility defines the condition where there is no movement.

Briefly, the samples were mixed and 10 µL of each sample was placed on a glass slide and

cover with a 22-mm×22-mm cover slip. Sperm motility was assessed using a sperm motility analyzer (VT–Sperm 3.1). Ten individual fields were observed randomly for sperm motility. Motility parameters such as average path velocity (VAP, μm/s), straight line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), and linearity (LIN, %) were also checked. At least, 200 spermatozoa were evaluated for each sample.

Evaluation of Acrosome Reaction

Acrosomal assessment was done by FITC-PSA (L0770, Sigma Aldrich, Germany) staining. Each sample was exposed to 1 mg/mL of FITC-PSA for 30 minutes and then to 10 μg/mL of DAPI (D9542, Sigma Aldrich, Germany) for 5 minutes at room temperature. Thereafter, the cells were monitored under a fluorescent microscope (Olympus BX51, Japan). The nucleus and acrosome were stained in blue and green with DAPI and FITC-PSA, respectively. Acrosome was considered reacted when the green fluorescent was limited to the equatorial segment or when the acrosome area was not observed.

Evaluation of Sperm Cell Viability

Eosin Y (E6003, Sigma Aldrich, Germany) was added to each sample (1:1), and the cells were observed after 30 seconds. Eosin can infiltrate dead cell membranes, and thereupon the head of the dead sperm appears in red. At least 200 cells were evaluated for each group, and the percentage of viable sperm was calculated.

Statistical Analysis

Data were analyzed with SPSS 16.0. The normal distribution of the data was assessed using the Kolmogorov–Smirnov test. The data were distributed normally. Then the statistical comparisons between the groups were done by using the one-way ANOVA. A P<0.05 was considered significant. The ANOVA was followed by the Tukey post hoc test, and a P<0.05 was considered significant for each post hoc test. The results were expressed as means±SDs.

Results

Thirty minutes' incubation of sperm in the medium containing NNC (CatSper channel inhibitor) and DPI (NOX5 inhibitor) reduced sperm viability in the progesterone-stimulated sperm (P<0.001 and P=0.03, respectively). The mortality rate was increased in comparison to the control group when the CatSper channel was inhibited by NNC (P=0.004) (figure 1).

The percentages of the progressive and nonprogressive motile sperm are shown in figure 2. NNC reduced sperm progressive motility relative to the control and progesterone groups (P<0.001). DPI could not change sperm motility in controlled condition, but it reduced progressive and increased nonprogressive motility in stimulated condition by progesterone in comparison to the progesterone group (P<0.001 and P=0.003, respectively). The addition of NNC and DPI caused a significant reduction in total motility, especially when the cells were exposed to progesterone (P<0.001).

Furthermore, VCL (μm/s), VSL (μm/s), VAP (μm/s), and LIN (%) were significantly reduced

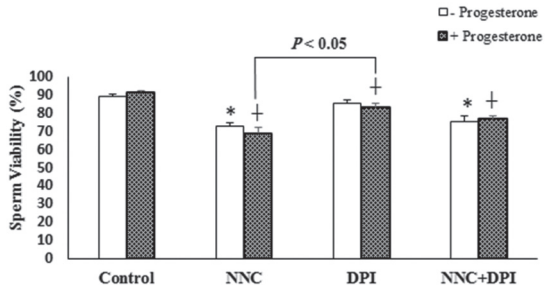

Figure 1: Graph depicts the effects of 1 μM of progesterone, 2 μM of NNC, and 1 μM of DPI as well as their combination on human sperm viability (%). *Significant difference between the groups without progesterone; ┼Significant difference between the groups containing progesterone; P<0.05 was considered significant.

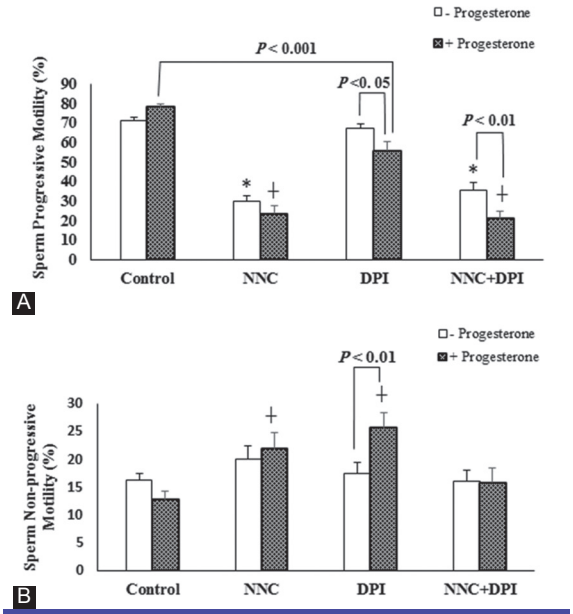

Figure 2: Graph depicts the effects of 1 μM of progesterone, 2 μM of NNC, and 1 μM of DPI as well as their combination on the (A) progressive and (B) nonprogressive motility of sperm. *Significant difference between the groups without progesterone; ┼Significant difference between the groups containing progesterone; P<0.05 was considered significant.

(P<0.001) in all the groups containing NNC or NNC plus DPI (table 1).

The percentage of acrosome-reacted sperm increased significantly by progesterone (from 8.7±0.68% in the control group to 21.9±1.7% in the progesterone group [P<0.001]). NNC and DPI did not have any significant effect in controlled condition, but they were able to completely block the effect of progesterone on AR (P<0.001) (figure 3).

Discussion

There is a direct relation between sperm motility and viability and male fertility. Both of these items are the most important factors assayed in semen analysis and have a determining role in the fertilization rate.[12] Many intracellular factors can affect sperm motility and survival such as intracellular calcium[13-15] and physiological ROS concentrations.[1,2]

However, the importance of NOX5 activity *vis-à-vis* sperm survival is not clear. According to our findings, it can be concluded that in the presence of progesterone, NOX5 activity plays a role in the maintenance of cell survival. It is not clear that this effect is related to NOX5 activity in ROS generation or to the other functions of the enzyme. Hampton and his colleagues[16] reported that 50 µM of H_2O_2 declined FAS-induced apoptosis in JurKat T-cells because of caspase activity inhibition. Nonetheless, it is well known that excess amounts of ROS induce apoptosis.[17]

As was mentioned above, intracellular calcium is another key factor in cell cycle and survival. It is proven that changes in calcium homeostasis are associated with cell death.[15] One report has shown that NNC reduces proliferation and induces apoptosis in leukemia cell lines.[18] In the present study, we showed that NNC decreased sperm cell viability. Since the main pathway for calcium influx in the human sperm is the CatSper channel,[10,19-21] it is quite reasonable to conclude that calcium entrance through the CatSper channel has a significant role in the intracellular calcium homeostasis and, thus, sperm survival.

In the current study, we evaluated both motility and AR as parameters expressing the fertilizing capacity of sperm. Comparing viability and total motility (figures 1 and 2 A, B) showed that all the live sperm were motile in the groups containing progesterone. Hence, progesterone could not induce a more significant increase in the percentage of the motile sperm. It has been clearly demonstrated that progesterone controls almost all aspects of human sperm function.[22,23] There are several reports that have shown that progesterone can induce sperm hyperactivation.[24,25] Nevertheless, some studies have shown that even increasing the concentration of progesterone from 1 nM to 10 µM could not induce sperm hyperactivation.[20,26] On the other hand, it has been proven by several studies that progesterone stimulates calcium influx into the spermatozoa through the CatSper channel.[9,10,27]

The addition of 500 nM of progesterone to the bath solution of sperm can considerably increase the amplitude of the CatSper channel's calcium current.[9,11] This progesterone-activated current can be blocked completely by NNC.[9] Moreover, research has shown that any mutation in CatSper channels prevents sperm hyperactivated motility and results in complete male infertility.[28-31] However, the mechanism whereby CatSper disruption or mutations leads

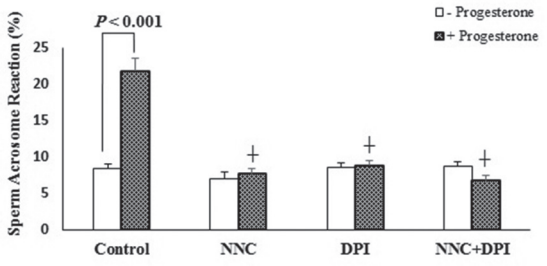

Figure 3: Graph depicts the effects of 1 µM of progesterone, 2 µM of NNC, and 1 µM of DPI as well as their combination on the percentage of the acrosome action of the human sperm. ┼Significant difference between the groups containing progesterone; P<0.001 was considered significant.

Table 1: Comparison of sperm motility parameters, curvilinear velocity (VCL, µm/s), straight line velocity (VSL, µm/s), average path velocity (VAP, µm/s), and linearity (LIN, %) between the different experimental groups after 30 minutes of incubation

Motility parameters	Without progesterone				With progesterone (1 µm)			
	Control (Ham's F-10)	NNC (2 µm)	DPI (1 µm)	NNC+DPI	Ham's F-10	NNC	DPI	NNC+DPI
VCL (µm/sec)	151.5±5.7	42.5±0.2*	144.8±9.4	46.9±12.4*	161.3±9.5	48.6±12.7┼	138.1±11.8	59.1±16.4┼
VSL (µm/sec)	82.5±4.9	17.1±3.3*	75.2±7.1	22.09±5.8*	78.8±6.9	18.6±4.1┼	64.8±8.1	19.3±6.04┼
VAP (µm/sec)	96.6±5.2	22.1±5.0*	84.9±7.9	25.8±6.7*	89.3±7.7	23.9±6.6┼	73.6±8.6	23.3±7.02┼
LIN (%)	52±3	33±2*	47±3	32±3*	43±2	31±3┼	40±4	19±2┼

*Significant difference between the groups without progesterone; ┼Significant difference between the groups containing progesterone; Results are expressed as means±SDs. P>0.001 was considered significant

to male infertility has not been determined. Another report has shown that the CatSper channel is required for calcium entry and control of sperm hyperactivation.[32]

The reference values considered as the hyperactivated state for the human sperm are VCL>150 µm/s and LIN<50%.[33] In our study, the VCL and LIN of those sperm which were incubated in progesterone were 161±9.5 µm/s and 43±2.4%, respectively. However, these values of motility in the progesterone group were not significantly different from those of the control group. On the other hand, AR was induced by progesterone. Based on these findings, in this study, we referred to the effect of progesterone on sperm as "the stimulation effect". In our experiment, the remarkable decrement in sperm motility by NNC underscores the importance of CatSper channel activity in sperm motility. In addition, we showed that the CatSper channel played a role even in the absence of progesterone. This finding is in agreement with other reports which applied the patch clamp technique.[9,11] To be more exact, these studies have demonstrated that even in the absence of progesterone, the CatSper channel is calcium-sensitive and there is a calcium current through the channel.

In regard to the NOX5 enzyme, DPI did not have any significant effect on sperm motility in the control group, but it reduced progressive motility and increased nonprogressive sperm motility in the presence of progesterone (figure 2).

It has been proven that adding NADPH to the sperm medium causes an increase in the tyrosine phosphorylation.[34] Moreover, it has been shown that there is a correlation between sperm ROS generation and tyrosine phosphorylation pattern.[35] Therefore, it can be concluded that redox activity plays a role in capacitation events such as progressive motility. Additionally, the incubation of sperm with 100 µM of H_2O_2 for 10 minutes caused an increase in the total percentage of sperm in the swim-up suspension.[4] It has been proven that this effect is due to NOX5 enzyme activity because the incubation of spermatozoa with DPI blocked this stimulatory effect.[4] Indeed, other studies have shown that adding either catalase or SOD will result in the loss of sperm fertilizing ability.[34,36]

Our finding suggests that NOX5 activity in the absence of progesterone does not have any effect on motility. However, under the same condition, CatSper may have a determining role in sperm motility. We showed that when sperm were stimulated by progesterone, the role of NOX5 was more prominent.

As was mentioned in the introduction, calcium activates the NOX5 enzyme. So it is likely that the CatSper channel affects sperm motility either directly or indirectly. For instance, it can be assumed that calcium stimulates NOX5 activity and ROS generation. Calcium and ROS can stimulate the signaling pathways that are involved in sperm motility. Therefore, we conclude that probably the actions of CatSper and NOX5 are somewhat linked together.

We showed that 1 µM of progesterone induced sperm AR. Similarly, other studies have also shown the same effect of progesterone on the AR of the human sperm.[20,22] In the present study, progesterone-induced AR was inhibited by either DPI, NNC, or DPI+NNC. Therefore, it can be assumed that the effect of progesterone on AR induction depends on the CatSper channel and NOX5 activity.

Although the present study was meticulously prepared, there were some unavoidable limitations. In general, because of the importance of the NOX5 enzyme and the CatSper channel in sperm physiology, more pharmacological and electrophysiological evidence is needed to clarify their role in sperm function and capacitation. For instance, the results of this study would have been confirmed further if we had measured calcium entrance to the cell via relevant techniques such as the patch clamp. In this way, the interactive functions of the NOX5 enzyme and the CatSper channel could have been examined more precisely.

Conclusion

Progesterone did not change sperm motility and viability, but it significantly induced AR. The CatSper channel plays a very important role in motility and viability either in the presence or in the absence of progesterone.

However, the effect of NOX5 activity on sperm motility and viability is more important when sperm are stimulated by progesterone. Based on the prominence of NNC in the reduction of sperm motility and viability and also given the role of progesterone in the regulation of calcium entry into sperm through the CatSper channel, we hypothesized that progesterone stimulated calcium entrance through CatSper and calcium activated NOX5 activity. Be that as it may, more studies are necessary to clarify the exact relationship between the activities of NOX5 and the CatSper channel as regards the stimulated function of the human sperm.

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Conflict of Interest: None declared.

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