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Original Article

Comparison between Inhibition of CatSper and KSper Channels with NNC 55-0396 and Quinidine on Human Sperm Function

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What's Known

- Several ions affect the function of human sperm. Calcium ion is the best known. Inhibition of the cation calcium channel of sperm disrupts sperm motility, viability, and fertilization capability.
- Specific potassium channels were identified in human spermatozoa. Opening of these channels results in potassium efflux and membrane hyperpolarization.

What's New

- Inhibition of calcium and potassium channels of sperm by NNC 55-0396 and quinidine, respectively, increase mortality and inhibit motility and kinematics of human sperm.
- Inhibition of sperm potassium channels without effect on intracellular calcium, inhibits sperm motility and increases mortality rate. Sperm potassium channels are as crucial as calcium channels.

Abstract

Background: Calcium enters human sperm through the "Cation Channel of Sperm" (CatSper), while potassium ions exit via the sperm potassium channel (KSper). These two channels regulate intracellular calcium concentration ($[Ca^{2+}]_i$) and membrane potential. Our study aims to investigate and compare the contributions of these channels in capacitated sperm function.

Methods: This study was conducted at the Physiology Department of Shiraz Medical School in 2022. Thirty-six semen samples were washed, and the supernatants were discarded. Then, 0.5 mL of modified-supplemented Earle's balanced salt solution (M-sEBSS) was added to the sediments. Samples were incubated at 37 ℃ and 5% CO_2. The motile spermatozoa were aspirated after swimming up, and the samples were diluted with M-sEBSS to 10×10^6 spermatozoa per mL. The samples were divided into four groups: control, solvent (dimethyl-sulphoxide (DMSO), NNC55-0396 (NNC) (10 µM), and quinidine (100 µM) as CatSper and KSper blockers, respectively. Sperm kinematics were evaluated by a computer-assisted sperm analyzer. The samples were stained with Eosin-Y and FITC-PSA to assess sperm viability and acrosomal reaction. The $[Ca^{2+}]_i$ was examined by flow cytometry using Fluo-3AM. The parametric and non-parametric data were analyzed using one-way ANOVA and Kruskal-Wallis test.

Results: NNC and quinidine significantly decreased progressive sperm motility (P=0.001) and reduced sperm kinematics (P=0.001). NNC but not quinidine significantly decreased sperm survival (P=0.001), reduced $[Ca^{2+}]_i$ in live spermatozoa (P=0.05), and induced the acrosomal reaction (P=0.012).

Conclusion: Inhibition of KSper without effect on $[Ca^{2+}]_i$ can inhibit sperm motility and increase mortality rate. It seems that the function of KSper is as vital as CatSper in human sperm physiology.

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Keywords ● Calcium channels ● Potassium channels ● NNC 55-0396 ● Quinidine

Introduction

The ionic channel activities and ionic flux are the main determining factors of membrane voltage. During human sperm activation and capacitation, the membrane permeability to these ions

changes, causing an alteration in the voltage of the sperm membrane. Ca^{2+}, K^{+}, H^{+}, Na^{+}, and Cl^{-} ion channels were identified in human sperm.[1] Activation of sperm is accompanied by membrane depolarization because of the influx of Na^{+} and Ca^{2+},[2] and the efflux of Cl^{-} ions.[3] However, membrane hyperpolarization is related to potassium efflux.[4] Besides the effect of ionic transport on the membrane potential,[5] intracellular ionic changes within the normal physiological range activate many signaling cascades essential for sperm capacitation, acrosomal reaction, and fertilization.[6] Among these ions, Ca^{2+} is crucial in numerous sperm functions.[7]

The primary calcium channel in the human sperm membrane is the "Cationic Channel of Sperm" (CatSper). It contains α, β, δ, γ, ε, ζ, and an EF-hand calcium-binding domain-containing protein 9 (EFCAB9) subunits. The α subunit has six transmembrane domains and forms the channel pore. The CatSper channel is activated in an alkaline environment.[2] The efflux of protons from the cell is a significant activator of CatSper. The whole-cell patch-clamp electrophysiology technique unveiled a proton conductance that activates upon depolarization. This conductance occurs through a voltage-gated proton channel in the principal piece, aptly named HV1.[8]

CatSper and HV1 are mildly voltage sensitive, and membrane depolarization creates ideal conditions for channel opening. It is worth mentioning that Na^{+} influx through the Depolarizing Channel of Sperm (DSper), a member of temperature-activated channels or transient receptor potential cation channel subfamily V member 4 (TRPV4), may remarkably affect membrane depolarization.[9]

On the other hand, endogenous plasma membrane endocannabinoids suppress CatSper activity. A progesterone-sensitive membrane lipid hydrolase in the principal piece metabolizes these endocannabinoids. Consequently, progesterone enhances the calcium current through the CatSper channel.[2]

The sperm potassium channel (KSper) restores cell membrane potential and even membrane hyperpolarization. The Slowpoke (SLO) family of K^{+} channels mediates the major KSper currents in mammals, including humans. Only SLO1 and SLO3 were identified in human spermatozoa. SLO1 channels are sensitive to membrane depolarization and intracellular Ca^{2+} concentration, whereas SLO3 channels exhibit sensitivity to intracellular alkalinization.[10] Thus, the activity of CatSper and Hv1 stimulates KSper opening and potassium efflux, resulting in membrane hyperpolarization that could deactivate the voltage-dependent channels such as Hv1 and CatSper.[1, 2] The activity of KSper can regulate the driving force for Ca^{2+} influxes from intracellular or extracellular resources by controlling the membrane voltage potential.[10] Therefore, the KSper can indirectly regulate intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and, as a result, sperm function.[1] Nevertheless, sperm capacitation and hyperactivation do not occur without a significant increase in $[Ca^{2+}]_i$ [2] and alkalization.[11] Some researchers believe that progesterone is a key factor in facilitating CatSper opening and inhibiting KSper, resulting in sperm capacitation.[12] However, other research emphasizes sperm hyperpolarization for capacitation and hyperactivation.[4, 10] Furthermore, KSper blockade or impairment leads to improper depolarization of sperm membrane potential and reduces fertility in men and even failure in *in-vitro* fertilization (IVF). Sperm physiology changes during capacitation in KSper-mutated sperm cells.[10]

Although substantial evidence exists regarding the roles of CatSper and KSper in human sperm physiology, several questions remain unanswered regarding the relative significance of these two channels in sperm function. In our study, NNC 55-0396 (NNC) and quinidine were employed as inhibitors for the CatSper and KSper channels, respectively, to investigate and compare the contributions of these channels in capacitated sperm viability and function.

Materials and Methods

Reagents and Chemicals

All chemicals were purchased from Sigma-Aldrich (Germany) except NNC, which was purchased from Cayman Chemical (USA). A stock solution of quinidine (Sigma-Aldrich 22600, Germany) (60 mM), Fluo3-AM (Sigma-Aldrich, 73881 Germany) (1 mM), and Pluronic F-127 (Sigma-Aldrich P2443, Germany) (15 mM) was prepared separately in dimethyl sulphoxide (DMSO) (Sigma-Aldrich D2650, Germany). Pisum sativum agglutinin labeled with fluorescein isothiocyanate (PSA-FITC) (Sigma-Aldrich L0770, Germany) and propidium iodide (PI) (Sigma-Aldrich P4170, Germany) were dissolved in phosphate buffer saline (Sigma-Aldrich P4417, Germany) with concentrations of 1 mg/mL and 1.5 mM, respectively. The NNC (Cayman Chemical CAY17216, USA) stock solution was prepared in distilled water (600 µM). All stock solutions were saved in a -20 °C freezer. They were melted and diluted before use.

Sperm Incubation Medium

The sperm incubation medium was modified-supplemented Earle's balanced salt solution (M-sEBSS), containing NaCl (Sigma-Aldrich S3014, Germany) (6.8 g), $NaHCO_3$ (Sigma-Aldrich S5761, Germany) (2.2 g), $NaH_2PO_4 \cdot H_2O$ (Sigma-Aldrich 71507, Germany) (0.14 g), KCl (Sigma-Aldrich P9541, Germany) (0.4 g), $MgSO_4 \cdot 7H_2O$ (Sigma-Aldrich M2773, Germany) (0.20 g), $CaCl_2 \cdot H_2O$ (Sigma-Aldrich C5670, Germany) (0.23 g), D-glucose (Sigma-Aldrich G7021, Germany) (1.0 g), Na-pyruvate (Sigma-Aldrich P5280, Germany) (0.30 g), Na-lactate (Sigma-Aldrich L4263, Germany) (19 mM) and 0.3% human serum albumin (Sigma-Aldrich A9731, Germany) per liter.[13]

Sample Collection and Preparation

This study was conducted at the Physiology Department of Shiraz Medical School in 2022. Thirty-six normal semen samples were collected from men who were 38.4±1.1 years old on average and referred to the Shiraz Fertility Center. These samples were obtained after 3 to 6 days of sexual abstinence. Following the liquefaction process (which took 15-30 min at 37 ºC and 5% CO_2), preliminary semen analyses were conducted. Only samples that met the standard criteria for normozoospermia—specifically, sperm counts ≥60×10^6 spermatozoa per mL and total motility ≥42%—were included in the study.[13] The donors did not have any specific diseases, and they did not take any supplements or medication; none of them were cigarette or alcohol consumers. The Research Ethics Committee at Shiraz University of Medical Sciences–approved the study protocol (IR.SUMS.REC.1401.079).

Sperm Preparation and Study Design

Semen samples were washed with sperm-capacitating medium M-sEBSS containing $NaHCO_3$ (2.2 g) and 0.3% human serum albumin per liter. For this, 1.5 mL of medium was added per 1 mL of semen to the falcon tubes containing the semen samples and stirred slowly, then centrifuged for 10 min at 1200-1500 rpm. The supernatants were discarded. Half mL of M-sEBSS was added to the remaining sediments of each sample. Samples were incubated at 37 ºC and 5% CO_2. The motile spermatozoa swam up and were aspirated. Then, spermatozoa were diluted with the medium to 10×10^6 spermatozoa per mL. The samples were divided into four groups: control, solvent (DMSO, 0.02%), NNC (10 µM), and quinidine (100 µM). All experimental groups were incubated at 37 ºC and 5% CO_2 for 10 min.

Assessment of Kinematics of Sperm

After incubation, a wet preparation with approximately 20 µm deep was prepared on a pre-warmed (37 ºC) slide and coverslip for each replicate (N=25). After stopping the drifting (about 60 seconds), slides were examined with a video test sperm analyzer (ArgusSoft, Ltd. Russia) equipped with an Olympus CX41 (Japan) phase-contrast microscope at a ×200 magnification optic. At least 200 motile sperm per replicate were assessed. Sperm were categorized into progressive, non-progressive, and immotile sperm.[14] Kinematics parameters, straight-line (rectilinear) velocity (VSL, µm/s), average path velocity (VAP, µm/s), curvilinear path velocity (VCL, µm/s), linearity of a curvilinear path (LIN) and amplitude of lateral head displacement (ALH) of sperm were checked out.

Assessment of Viability of Sperm

To assess sperm survival, the sample was gently stirred, and 10 µL of it was mixed with the same volume of eosin Y (Sigma E6003, Germany, n=25). After 30 seconds, a thin smear of 10 µL of this mixture was prepared and covered with a 22×22 mm coverslip. At least 200 spermatozoa per replicate were evaluated under a phase-contrast microscope at a magnification of ×400. The heads of dead spermatozoa appeared red, whereas those of live spermatozoa were shiny white.

Assessment of $[Ca^{2+}]_i$ by Flow Cytometry

Briefly, the aliquots of sperm cells (1×10^6 sperm cells/mL in M-sEBSS medium, n=6) were incubated with 10 µM fluorescent Ca^{2+} indicator Fluo-3 AM (Sigma 73881, Germany) containing 0.08% pluronic F-127 (Sigma P2443, Germany) at dark for 30 min at 37 °C and 5% CO_2. Excess dye was removed by centrifugation at room temperature at 500×g for 5 min. Five µM of PI (Sigma P4170, Germany) was applied just 5 min before assaying to evaluate the viability of sperm cells. After that, 50,000 spermatozoa were assessed by flow cytometry (FACSCalibur; BD: USA). Fluorescence was excited at 506 nm, and emission was recorded at 525 nm. The results were analyzed using FlowJo™ (USA) version 10. The cells were divided into four quadrants according to their viability and $[Ca^{2+}]_i$. Dead spermatozoa with low $[Ca^{2+}]_i$, dead spermatozoa with high $[Ca^{2+}]_i$, live spermatozoa with high $[Ca^{2+}]_i$ and live spermatozoa with low $[Ca^{2+}]_i$ belonged to the 1^{st}–4^{th} quadrants (Q1-Q4), respectively. The mean Fluorescence intensity (MFI) was calculated.

Assessment of Acrosome Status

Pisum sativum agglutinin (PSA) labeled

with fluorescein isothiocyanate (FITC) (PSA-FITC) was used to assess acrosome status. Meanwhile, as described below, we used the hypo-osmotic swelling (HOS) buffer to evaluate acrosome reaction only in live spermatozoa. Live sperm with a healthy membrane swell, and the sperm's tail becomes curly in the HOS solution. The HOS buffer contains 0.735 g of sodium citrate dihydrate (Sigma-Aldrich C8532, Germany) and 1.351 g of D-fructose (Sigma-Aldrich F3510, Germany) dissolved in 100 mL of distilled water. The HOS buffer was diluted 1:1 with distilled water before use. At the time of the experiment, 100 µL of each sample was added to 1 mL of diluted HOS buffer and incubated for 30 min. The samples were centrifuged at 1200 rpm for 5 min, then the supernatant was discarded, and the resulting sediment was resuspended by pipetting and used to prepare a smear.

A smear of 10 µL of each sample was prepared and placed at room temperature to dry completely, then put in an ethanol (Razi, Iran) container for 15-30 min. At the end, the slides were removed from the ethanol pool and exposed to room air; after drying, they were stored at -20 ˚C.

The slides were removed from the freezer and kept at room temperature to assess the acrosome status. Sperm smears were stained by 50 µg/mL of PSA-FITC and stored at room temperature in a dark container for 60 min. Then, the slides were washed with distilled water for 15 min. Finally, the slides were examined by fluorescent microscopy with a FITC filter (450-490 nm) at X1000 magnification optic (BX51; Olympus, Japan). At least 200 sperm were assessed in each group. Live sperm had bent and curly tails. The head of sperm with intact acrosome was bright green and uniformly fluorescing. In contrast, the head of acrosome-reacted sperm was shiny green only in the equatorial segment, and there was no fluorescing stain in the acrosome region.[13]

Statistical Analysis

Data were analyzed using IBM SPSS software (version 26, USA). The Kolmogorov-Smirnov test was used as a normality test. The one-way ANOVA and Tukey's *post hoc* test were used for normal-distributed data (the data of sperm motility, kinematics, and viability). The data were reported as mean±SEM, and P≤0.05 was considered a significant difference. The Kruskal-Wallis and Dunn's test was used for acrosome reaction and $[Ca^{2+}]_i$ assessment. These data were reported as median (Q1-Q3), and P≤0.05 was considered as a significant difference.

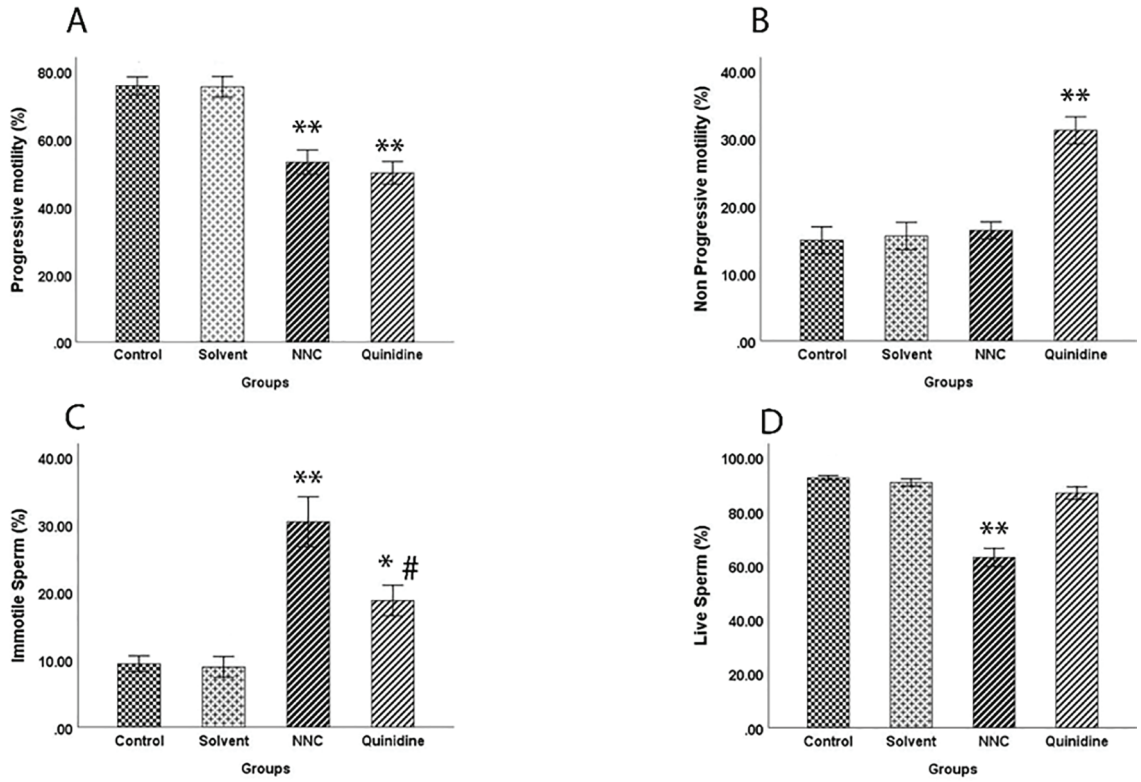

Figure 1: Graphs represent the effects of NNC and quinidine on the motility and viability of sperm. The effects of NNC and quinidine on A) Progressive motility (%), B) Non-progressive motility (%), C) Immotile sperm (%), and D) Viability of sperm. Data represent the mean±SEM. ** and * shows significant difference with control and sham groups (**P=0.001, and *P=0.034), and #shows significant difference between NNC and quinidine groups (P=0.004). n=25 in each group

Results

The Effect of NNC and Quinidine on Sperm Motility and Survival

NNC significantly decreased progressive sperm motility and increased immotile sperm (P=0.001), while there was no significant change in non-progressive sperm in the NNC group. Meanwhile, quinidine significantly reduced progressive motility and increased non-progressive motility (P=0.001) and immotile sperm (P=0.034) (figures 1A-C).

The survival of sperm was assessed using the eosin-Y staining method. The percentage of live sperm in the control group was 92.55±0.84. NNC reduced sperm survival to 63.02±3.39% (P=0.001) (figure 1D).

The Effect of NNC and Quinidine on the Kinematics of Motile Sperm

Generally, NNC and quinidine reduced sperm kinematics. However, their effects on all parameters were not the same. NNC and quinidine significantly decreased VSL from 103.13±4.97 µm/s in control to 71.91±3.42 and 52.52±4.24 µm/s, respectively (P=0.001) (figure 2A). However, their diminishing effect on VCL was similar (P=0.001 for NNC and P=0.001 for quinidine) (figure 2B). VAP was reduced in NNC and quinidine-containing groups by 31% and 48%, respectively (P=0.001) (figure 2C). NNC reduced ALH from 2.12±0.09 µm in the control group to 1.60±0.08 µm (P=0.001) (figure 2D). LIN showed a significant decrease in the quinidine group (P=0.001) (figure 2E) because LIN results from VSL divided to VCL, and quinidine prominently reduced VSL relative to NNC.

The Effect of NNC and Quinidine on $[Ca^{2+}]_i$

Flow cytometry analysis of $[Ca^{2+}]_i$ and viability staining by Fluo-3 AM and PI showed a significant decrease (P=0.045) (15%) in $[Ca^{2+}]_i$ of live spermatozoa (Q3) in the NNC-containing group (figure 3 and table 1). The mean fluorescence intensity (MFI) of Fluo-3 AM was calculated and revealed a significant decrease (P=0.037) when the cells were exposed to NNC. The median (Q1-Q3) of the data is reported in table 1.

Effect of NNC and Quinidine on the Acrosomal Reaction

A smear of sperm samples exposed to hypo-osmotic buffers was prepared and stained by PSA-FITC. During the assessment of acrosome reaction, HOST was employed to distinguish between viable and non-viable sperm. Sperm exhibiting a bright and uniformly fluorescing head were identified as live with intact acrosomes. In contrast, sperm that had undergone acrosome reaction displayed only a fluorescing band at the equatorial segment or no fluorescence in the acrosomal region.

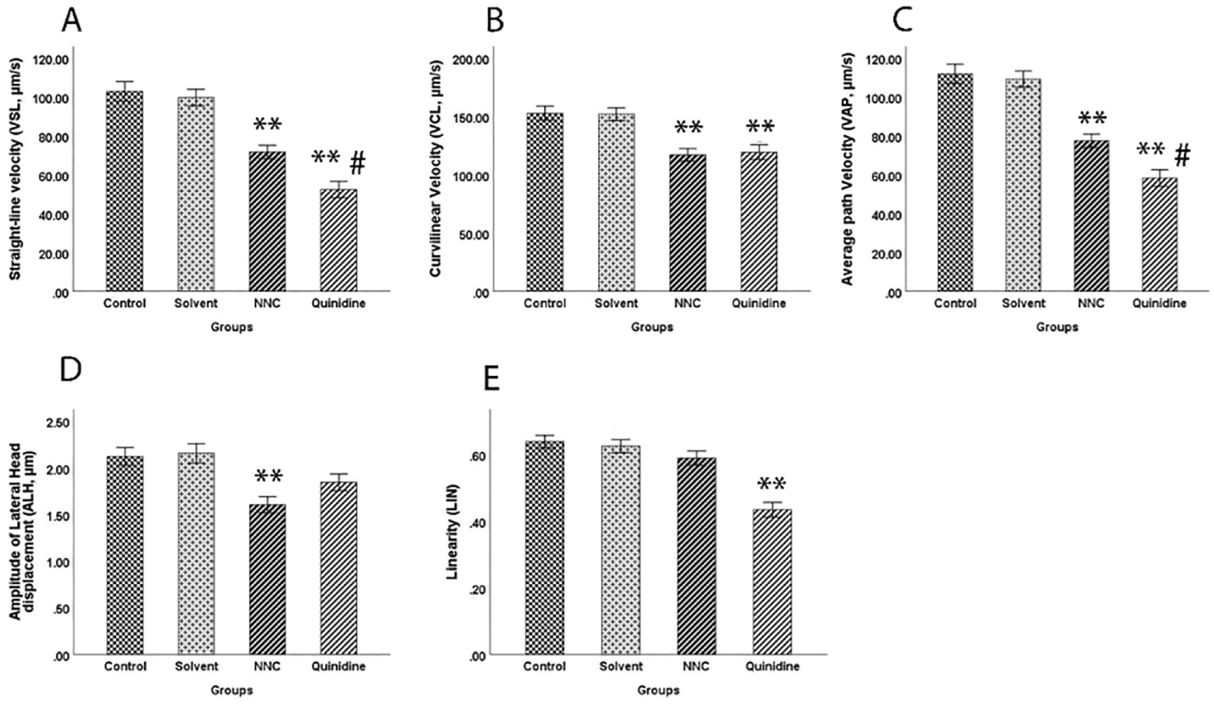

Figure 2: Graphs represent the effects of NNC and quinidine on sperm kinematics. The effects of NNC and quinidine on A) Straight-line velocity (VSL), B) Curvilinear velocity (VCL), C) Average path velocity (VAP), D) Amplitude of lateral head displacement (ALH), and E) Linearity (LIN). Data represent the mean±SEM. The identical letters are not significantly different from each other. **Shows significant difference with control and sham groups (**P=0.001), and #shows significant difference between NNC and quinidine groups (P=0.01). n=25 in each group

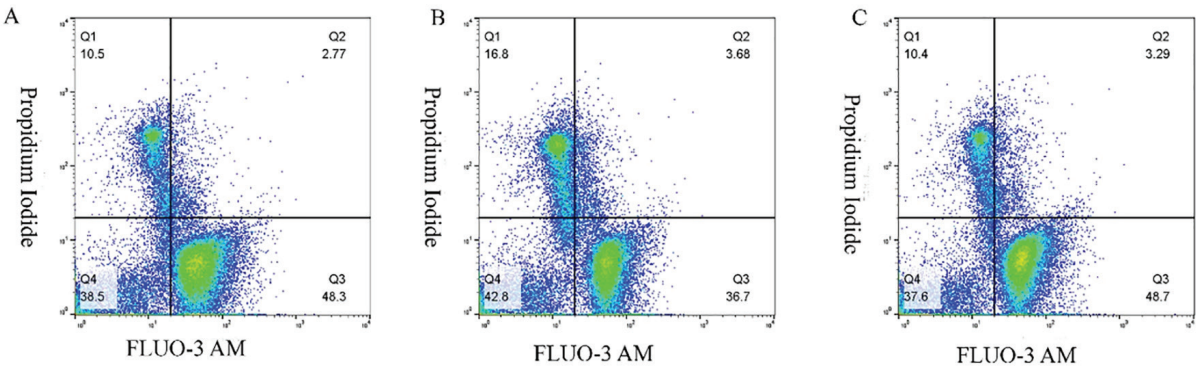

Figure 3: Assessment of $[Ca^{2+}]_i$ and viability performed by Flow cytometry method, using Fluo-3 AM and PI. A) Control, B) NNC, and C) quinidine groups

Table 1: The Effects of NNC and quinidine on $[Ca^{2+}]_i$ and mean fluorescent intensity

	Control Median (Q1-Q3)	NNC Median (Q1-Q3)	Quinidine Median (Q1-Q3)	P value
Dead spermatozoa with low $[Ca^{2+}]_i$	4.69 (0.66-10.92)	10.05 (2.39-20.35)	2.62 (1.17-6.20)	0.42
Dead spermatozoa with high $[Ca^{2+}]_i$	3.34 (0.48-6.93)	9.14 (2.21-20.20)	5.11 (0.89-15.35)	0.49
Live spermatozoa with high $[Ca^{2+}]_i$	42.25 (32.82-49.77)	24.30 (17.02-36.90) * (P=0.045)	44.25 (25.12-47.50) (P=0.4)	0.05
Live spermatozoa with low $[Ca^{2+}]_i$	47.35 (41.12-57.02)	55.10 (42.02-62.00)	50.85 (37.22-60.85)	0.75
Mean Fluorescent Intensity (MFI)	26.30 (17.57-31.60)	14.60 (12.72-19.17)** (P=0.037)	19.75 (17.55-29.10) (P=0.3)	0.05

Values are presented as median (Q1-Q3). n=6 in each group; P values show a comparison with the control group.

Table 2: The Effects of NNC and quinidine on acrosomal reaction

	Control Median (Q1-Q3)	Solvent Median (Q1-Q3)	NNC Median (Q1-Q3)	Quinidine Median (Q1-Q3)
Acrosome Reaction	4.86 (4.54-6.45)	3.77 (3.24-5.43)	8.25 (6.61-17.10)	5.26 (2.56-5.78)
P value		P=0.28	P=0.04 *	P=0.39

Values are presented as median (Q1-Q3) (n=5). P values show a comparison with the control group.

In the control and solvent groups, 5.37±0.44% and 4.22±0.60% of sperm were acrosome reacted. Meanwhile, NNC (11.83±2.35%), but not quinidine (4.39±0.75%), induced acrosome reaction significantly (P=0.012). The median (Q1-Q3) of the data is reported in table 2.

Discussion

In this study, we showed that the blockage of the CatSper by 10 µM NNC and inhibition of KSper opening by 100 µM quinidine caused a significant reduction in the percentage of progressively motile sperm, accompanied by an increment in immotile sperm. Despite NNC, which reduced sperm survival, quinidine had a non-significant effect on vitality. A significant decrease in $[Ca^{2+}]_i$ was observed by NNC but not quinidine. As well, NNC and quinidine decreased sperm kinematics.

The electrophysiological study showed that NNC at 2 µM concentration inhibited the calcium current through CatSper.[15] It was reported that 2 µM,[16-18] and 10 µM of NNC[19] reduced the progressive movement of sperm. However, NNC could not wholly reduce the percentage of progressive motile sperm, and about 70% of sperm were still motile after NNC treatment. Meanwhile, the percentage of dead sperm was greater than that of immotile sperm by NNC. The impact of NNC on sperm motility and viability appears to vary over time,[16-18] and after some time, all sperm may become immotile. However, similar results were obtained in the group treated with 100 µM quinidine (KSper blocker) at the same incubation time. Millimolar concentrations of quinidine immediately immobilize human sperm.[20] The exposure of sperm to 500 µM quinidine for 2 hours significantly increases the number of dead and abnormal sperm.[21] Therefore, a high dose of quinidine has a toxic effect on human sperm. Meanwhile, it was recorded that the sperm of mice lacking SLO3 channels had no progressive movements.[22]

NNC and quinidine also decreased sperm kinematics. There is a direct linear relationship (R^2>0.9) between some kinematics parameters of sperm (VCL and ALH) with $[Ca^{2+}]_i$.[23] We observed that NNC (10 µM) reduces VSL, VCL, VAP and ALH of motile sperm. Others reported that 2 µM and 10 mM NNC reduce VSL and VAP. As well as 2 µM, 10 mM, and 20 mM NNC reduce LIN.[16, 19] In addition, 2 µM NNC reduces VCL[16, 18] and ALH.[18] However, some authors reported that VSL, VAP, and LIN did not change by 2 µM NNC,[18] and 10 mM NNC had no effect on ALH and VCL.[19] So far, no study has investigated the effect of quinidine on sperm kinematics; however, we found that quinidine decreased VSL, VCL, VAP, and LIN. Compared to NNC, quinidine can cause a more significant decrease in VSL and VAP. In one study, an analog of quinidine (quinine) was used, and they found that 20 µM quinine can increase ALH and reduce LIN and ASL. As well, 20 µM quinine increased VCL by 10-20%. Moreover, 125 µM quinine in the presence of Ca^{2+} decreased VSL and increased VCL. It also reduced LIN and increased ALH in the presence and absence of Ca^{2+}.[24]

Flucytometric analysis of $[Ca^{2+}]_i$ showed NNC, but not quinidine, reduced the percentage of live-high calcium sperm. On the other hand, regarding our findings, the spermostatic effect of CatSper and KSper blockers were almost similar at 10 min incubation time, and we found that quinidine, with no impact on $[Ca^{2+}]_i$, inhibited sperm motility. Although there is much evidence of the role of Ca^{2+} in sperm function, our results showed that inhibiting the potassium channel and preventing its efflux can similarly affect the inhibition of sperm motility. Some mechanisms can be explained to justify this event. In human sperm, exposure to an external alkaline pH could activate the Hv1 channel and extrusion of proton ions from the cell, increasing pH_i. The intracellular alkalization contributes to the activation of CatSper and SLO3 channels. The Ca^{2+} ions enter the sperm through CatSper and increase in $[Ca^{2+}]_i$ which is one of the critical stimulators of SLO3.[10] Activation of SLO3 leads to membrane hyperpolarization.[10] It has been proposed that membrane hyperpolarization provides a driving force for Na^+ entrance and consequently enhances the activity of Na^+/H^+ exchangers in mouse sperm. The extrusion of proton ions elevates the intracellular pH (positive feedback).[25] In human sperm, the proton mainly extrudes through Hv1; meanwhile, the CatSper opening is weakly voltage-dependent.[26] Therefore, membrane hyperpolarization may not significantly affect CatSper opening, but it makes a driving force for Na^+.

The membrane potential in non-capacitated human sperm is -40mV and reaches -50mV after capacitation.[27] This cellular hyperpolarization in capacitated spermatozoa depends on the opening of potassium channels. It is reported that blocking the SLO3 channel by quinidine prevents membrane hyperpolarization and simultaneously blocks the current through CatSper,[28] reducing the progressive motility of sperm and immotility. Thus, it is possible that the reduction of sperm motility in the quinidine-treated group was caused by the direct effect of quinidine on Catsper channels, not by the indirect impact and membrane hyperpolarization. The fluctuations of $[Ca^{2+}]_i$ are dependent on sperm membrane voltage. Using valinomycin as a potassium ionophore can eliminate the fluctuations of $[Ca^{2+}]_i$ caused by progesterone. Increasing the potassium concentration in the environment containing valinomycin can neutralize the effects of valinomycin.

Furthermore, using quinidine (300 µM) as a sperm potassium channel inhibitor completely inhibits $[Ca^{2+}]_i$ fluctuations.[28] Moreover, 2 µM NNC can inhibit the potassium current of sperm and depolarize the membrane voltage. [29] Therefore, in this way, NNC can also prevent $[Ca^{2+}]_i$ fluctuations. Furthermore, 10 µM quinidine reversibly reduces the influx of Ca^{2+} into the sperm.[21] Additionally, when there are no divalent ions such as Ca^{2+} and Mg^{2+} in the environment, monovalent ions such as Na^+, K^+, and Cs^+ can pass through the CatSper channel.[15, 30] Quinidine (0.3 mM) blocks the cesium current through the CatSper.[29] Therefore, some researchers have mentioned quinidine as a CatSper channel blocker.[31] As a result, quinidine can affect the fluctuations of $[Ca^{2+}]_i$ through several different pathways. We investigated the $[Ca^{2+}]_i$ in the quinidine-containing group to test the probability of the direct effect of quinidine on the CatSper channel and $[Ca^{2+}]_i$. Quinidine did not make a significant difference in $[Ca^{2+}]_i$. We concluded that the effect of quinidine on sperm motility was not by the reduction in $[Ca^{2+}]_i$. It is suggested that quinidine may prevent sperm hyperpolarization and affect other cellular ion balances. However, a hyperpolarization K^+ current was seen through CatSper at a membrane potential of more than -30 mV.[32]

Regarding the transport of K^+ through the inner membrane of mitochondria, it regulates the volume of the mitochondrial matrix, affects the respiratory rate, membrane potential, calcium transport, production of reactive oxygen species, and, in general, mitochondrial metabolism and energy homeostasis. Thus, it is suggested that quinidine can disrupt the metabolism of

mitochondria and homeostasis of sperm energy by blocking the influx of potassium into the cell, leading to changes in the kinematics of sperm and its immotility.[33]

Our survey on sperm viability showed a significant decrease after treatment of the cells with NNC. The spermicidal effects of NNC,[16-18,34,35] and quinidine[20] on human sperm were reported. Quinidine stabilizes the membrane by acting on an open Na^+ channel,[20] and NNC prevents the Ca^{2+} influx by blocking CatSper.[20, 29] Higher concentrations of quinidine than we used (2, 6, and 8 mM of quinidine) on human sperm were investigated. They showed that the spermicidal effect of quinidine was more potent than its spermostatic effect. Quinidine affects open-state Na^+ channels, L-type calcium channels, and potassium channels.[20, 29]

The NNC, but not quinidine, induced the acrosomal reaction in human sperm. A micromolar concentration (10 µM) of NNC was reported to cause an acrosomal reaction in human sperm[19] even in calcium-free environments.[36] In addition, 10 µM NNC increases $[Ca^{2+}]_i$[30] because NNC, as a weak base, raises the acrosome pH and releases Ca^{2+} from it. NNC is a highly lipophilic agent and easily passes through the membrane. It accumulates in intracellular acidic compartments (acrosome) and mediates the release of Ca^{2+} from them. Cytoplasmic alkalinization is not necessarily sufficient to induce the acrosomal reaction, whereas acrosome alkalinization is necessary to release Ca^{2+} from it, leading to the acrosomal reaction.[36] However, the effect of NNC on the increment of $[Ca^{2+}]_i$ may be transient, and even a temporary elevation in Ca^{2+} could induce the acrosome reaction.

In this study, we had some limitations. We didn't have the facilities to check changes in sperm voltage. Meanwhile, except for calcium, intracellular concentrations of other ions, such as Na^+ and K^+, and intracellular pH, were not evaluated in this experiment. These evaluations can show the exact mechanism of the quinidin's effect on sperm.

Conclusion

In conclusion, we showed that blocking Ca^{2+} and K^+ channels by NNC and quinidine significantly decreased sperm motility and kinematics parameters. Functions of potassium channels are essential as calcium channels and inhibition of potassium current disrupt sperm motility without a significant effect on $[Ca^{2+}]_i$. The changes in intracellular potassium and hydrogen ion concentrations and membrane potential can be evaluated to understand better the mechanisms involved in regulating sperm function.

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Authors' Contribution

S.K participated in the study conception and design and oversaw the operation of experimental protocols/data acquisition, interpretation of the data, and revision of the manuscript. AA.Z participated in the study conception and design, contributed to performing experimental methods, acquired and analyzed the data, and drafted the manuscript. M.H, N.F, and MH.N-E advised some experimental protocols, and interpretation of some obtained data and were involved in the critical revision of the manuscript. All the authors participated in the study design and approved the final manuscript for submission.

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References

1 Brown SG, Publicover SJ, Barratt CLR, Martins da Silva SJ. Human sperm ion channel (dys)function: implications for fertilization. Hum Reprod Update. 2019;25:758-76. doi: 10.1093/humupd/dmz032. PubMed PMID: 31665287; PubMed Central PMCID: PMCPMC6847974.

2 Rahban R, Nef S. CatSper: The complex main gate of calcium entry in mammalian spermatozoa. Mol Cell Endocrinol. 2020;518:110951. doi: 10.1016/j.mce.2020.110951. PubMed PMID: 32712386.

3 Turner KO, Meizel S. Progesterone-mediated efflux of cytosolic chloride during the human sperm acrosome reaction. Biochem Biophys Res Commun. 1995;213:774-80. doi: 10.1006/bbrc.1995.2197. PubMed PMID: 7654237.

4 Sanchez-Carranza O, Torres-Rodriguez P, Darszon A, Trevino CL, Lopez-Gonzalez I.

Pharmacology of hSlo3 channels and their contribution in the capacitation-associated hyperpolarization of human sperm. Biochem Biophys Res Commun. 2015;466:554-9. doi: 10.1016/j.bbrc.2015.09.073. PubMed PMID: 26381170.
5 Pinto FM, Odriozola A, Candenas L, Subiran N. The Role of Sperm Membrane Potential and Ion Channels in Regulating Sperm Function. Int J Mol Sci. 2023;24. doi: 10.3390/ijms24086995. PubMed PMID: 37108159; PubMed Central PMCID: PMCPMC10138380.
6 Lishko PV, Kirichok Y, Ren D, Navarro B, Chung JJ, Clapham DE. The control of male fertility by spermatozoan ion channels. Annu Rev Physiol. 2012;74:453-75. doi: 10.1146/annurev-physiol-020911-153258. PubMed PMID: 22017176; PubMed Central PMCID: PMCPMC3914660.
7 Mata-Martinez E, Sanchez-Cardenas C, Chavez JC, Guerrero A, Trevino CL, Corkidi G, et al. Role of calcium oscillations in sperm physiology. Biosystems. 2021;209:104524. doi: 10.1016/j.biosystems.2021.104524. PubMed PMID: 34453988.
8 Lishko PV, Botchkina IL, Fedorenko A, Kirichok Y. Acid extrusion from human spermatozoa is mediated by flagellar voltage-gated proton channel. Cell. 2010;140:327-37. doi: 10.1016/j.cell.2009.12.053. PubMed PMID: 20144758.
9 Mundt N, Spehr M, Lishko PV. TRPV4 is the temperature-sensitive ion channel of human sperm. Elife. 2018;7. doi: 10.7554/eLife.35853. PubMed PMID: 29963982; PubMed Central PMCID: PMCPMC6051745.
10 Lyon MD, Ferreira JJ, Li P, Bhagwat S, Butler A, Anderson K, et al. SLO3: A Conserved Regulator of Sperm Membrane Potential. Int J Mol Sci. 2023;24. doi: 10.3390/ijms241311205. PubMed PMID: 37446382; PubMed Central PMCID: PMCPMC10342799.
11 Matamoros-Volante A, Trevino CL. Capacitation-associated alkalization in human sperm is differentially controlled at the subcellular level. J Cell Sci. 2020;133. doi: 10.1242/jcs.238816. PubMed PMID: 31932506.
12 Mannowetz N, Naidoo NM, Choo SA, Smith JF, Lishko PV. Slo1 is the principal potassium channel of human spermatozoa. Elife. 2013;2:e01009. doi: 10.7554/eLife.01009. PubMed PMID: 24137539; PubMed Central PMCID: PMCPMC3789364.
13 World Health O. WHO laboratory manual for the examination and processing of human semen. 6th ed. Geneva: World Health Organization; 2021.
14 World Health O. WHO laboratory manual for the examination and processing of human semen. 5th ed ed. Geneva: World Health Organization; 2010.
15 Lishko PV, Botchkina IL, Kirichok Y. Progesterone activates the principal Ca2+ channel of human sperm. Nature. 2011;471:387-91. doi: 10.1038/nature09767. PubMed PMID: 21412339.
16 Keshtgar S, Ghanbari H, Ghani E, Shid Moosavi SM. Effect of CatSper and Hv1 Channel Inhibition on Progesterone Stimulated Human Sperm. J Reprod Infertil. 2018;19:133-9. PubMed PMID: 30167393; PubMed Central PMCID: PMCPMC6104424.
17 Ghanbari H, Keshtgar S, Kazeroni M. Inhibition of the CatSper Channel and NOX5 Enzyme Activity Affects the Functions of the Progesterone-Stimulated Human Sperm. Iran J Med Sci. 2018;43:18-25. PubMed PMID: 29398748; PubMed Central PMCID: PMCPMC5775990.
18 Maleki S, Keshtgar S, Ebrahimi B, Matavos-Aramyan H, Karbalaei N, Masjedi F. Effects of CatSper Stimulation and Inhibition by Progesterone and NNC on Human Sperm. Andrologia. 2023;2023:7826956. doi: 10.1155/2023/7826956.
19 Tamburrino L, Marchiani S, Minetti F, Forti G, Muratori M, Baldi E. The CatSper calcium channel in human sperm: relation with motility and involvement in progesterone-induced acrosome reaction. Hum Reprod. 2014;29:418-28. doi: 10.1093/humrep/det454. PubMed PMID: 24430778.
20 Moudgil P, Gupta A, Sharma A, Gupta S, Tiwary AK. Spermicidal efficacy of some membrane stabilizers. Pharmacology Reviews and Communications. 2002;12:241-8.
21 Alghazal SMA, Ding J, Hou P. Cytotoxic effect of Quinidine on testicular tissues, sperm parameters and Ca 2 level in mice sperms; as an option of male contraception. Advances in Life Science and Technology. 2015;30.
22 Santi CM, Martinez-Lopez P, de la Vega-Beltran JL, Butler A, Alisio A, Darszon A, et al. The SLO3 sperm-specific potassium channel plays a vital role in male fertility. FEBS Lett. 2010;584:1041-6. doi: 10.1016/j.febslet.2010.02.005. PubMed PMID: 20138882; PubMed Central PMCID: PMCPMC2875124.
23 Achikanu C, Pendekanti V, Teague R, Publicover S. Effects of pH manipulation, CatSper stimulation and Ca2+-store mobilization on [Ca2+]i and behaviour of human sperm. Hum Reprod. 2018;33:1802-11. doi: 10.1093/humrep/dey280. PubMed PMID: 30239776.

24 Yeung CH, Cooper TG. Effects of the ion-channel blocker quinine on human sperm volume, kinematics and mucus penetration, and the involvement of potassium channels. Mol Hum Reprod. 2001;7:819-28. doi: 10.1093/molehr/7.9.819. PubMed PMID: 11517288.
25 Hernandez-Garduno S, Chavez JC, Matamoros-Volante A, Sanchez-Guevara Y, Torres P, Trevino CL, et al. Hyperpolarization induces cytosolic alkalization of mouse sperm flagellum probably through sperm Na+/H+ exchanger. Reproduction. 2022;164:125-34. doi: 10.1530/REP-22-0101. PubMed PMID: 35900329.
26 Sun XH, Zhu YY, Wang L, Liu HL, Ling Y, Li ZL, et al. The Catsper channel and its roles in male fertility: a systematic review. Reprod Biol Endocrinol. 2017;15:65. doi: 10.1186/s12958-017-0281-2. PubMed PMID: 28810916; PubMed Central PMCID: PMCPMC5558725.
27 Patrat C, Serres C, Jouannet P. Progesterone induces hyperpolarization after a transient depolarization phase in human spermatozoa. Biol Reprod. 2002;66:1775-80. doi: 10.1095/biolreprod66.6.1775. PubMed PMID: 12021061.
28 Torrezan-Nitao E, Brown SG, Mata-Martinez E, Trevino CL, Barratt C, Publicover S. [Ca2+] i oscillations in human sperm are triggered in the flagellum by membrane potential-sensitive activity of CatSper. Hum Reprod. 2021;36:293-304. doi: 10.1093/humrep/deaa302. PubMed PMID: 33305795.
29 Mansell SA, Publicover SJ, Barratt CL, Wilson SM. Patch clamp studies of human sperm under physiological ionic conditions reveal three functionally and pharmacologically distinct cation channels. Mol Hum Reprod. 2014;20:392-408. doi: 10.1093/molehr/gau003. PubMed PMID: 24442342; PubMed Central PMCID: PMCPMC4004083.
30 Strunker T, Goodwin N, Brenker C, Kashikar ND, Weyand I, Seifert R, et al. The CatSper channel mediates progesterone-induced Ca2+ influx in human sperm. Nature. 2011;471:382-6. doi: 10.1038/nature09769. PubMed PMID: 21412338.
31 Alghazal SMA, Ding J. Calcium Channel Blocker (Quinidine) as a New Approach of Male Contraception: A Review. Advances in Life Science and Technology. 2015;29:57-62.
32 Zeng XH, Navarro B, Xia XM, Clapham DE, Lingle CJ. Simultaneous knockout of Slo3 and CatSper1 abolishes all alkalization- and voltage-activated current in mouse spermatozoa. J Gen Physiol. 2013;142:305-13. doi: 10.1085/jgp.201311011. PubMed PMID: 23980198; PubMed Central PMCID: PMCPMC3753610.
33 Laskowski M, Augustynek B, Kulawiak B, Koprowski P, Bednarczyk P, Jarmuszkiewicz W, et al. What do we not know about mitochondrial potassium channels? Biochim Biophys Acta. 2016;1857:1247-57. doi: 10.1016/j.bbabio.2016.03.007. PubMed PMID: 26951942.
34 Ghanbari H, Keshtgar S, Zare HR, Gharesi-Fard B. Inhibition of CatSper and Hv1 Channels and NOX5 Enzyme Affect Progesterone-Induced Increase of Intracellular Calcium Concentration and ROS Generation in Human Sperm. Iran J Med Sci. 2019;44:127-34. PubMed PMID: 30936599; PubMed Central PMCID: PMCPMC6423429.
35 Carlson EJ, Francis R, Liu Y, Li P, Lyon M, Santi CM, et al. Discovery and Characterization of Multiple Classes of Human CatSper Blockers. ChemMedChem. 2022;17:e202000499. doi: 10.1002/cmdc.202000499. PubMed PMID: 35644882; PubMed Central PMCID: PMCPMC9378630.
36 Chavez JC, De la Vega-Beltran JL, Jose O, Torres P, Nishigaki T, Trevino CL, et al. Acrosomal alkalization triggers Ca(2+) release and acrosome reaction in mammalian spermatozoa. J Cell Physiol. 2018;233:4735-47. doi: 10.1002/jcp.26262. PubMed PMID: 29135027.