

Research Article

Effects of CatSper Stimulation and Inhibition by Progesterone and NNC on Human Sperm

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Calcium ion enters the sperm through a specific calcium channel, CatSper. This voltage-sensitive channel is stimulated by intracellular alkalization and progesterone. This study is aimed at investigating the effects of CatSper inhibition or stimulation on sperm motility, viability, and sperm function regulators such as mitochondrial membrane potential (MMP), ATP, and reactive oxygen species (ROS) production. This study was performed on 30 semen samples of fertile volunteers, referred to Shiraz Fertility Center. The semen samples were diluted to 20×10^6 sperm/mL. The samples were divided randomly into control, solvent, progesterone ($10 \mu\text{M}$), NNC ($2 \mu\text{M}$), and NNC+progesterone groups. Sperm kinematics, viability, MMP, ATP content, and the amount of ROS production were assessed using VT-SPERM3.1, eosin staining, JC1 flow cytometry, bioluminescence, and chemiluminescence methods, respectively. Sperm viability and total and progressive motility were significantly decreased in the NNC and NNC+progesterone groups. The amplitude of lateral head displacement (ALH) and curvilinear velocity (VCL) was reduced in the NNC-containing groups. These parameters did not change in the progesterone group. ROS production by viable spermatozoa in the NNC and NNC+progesterone groups was significantly higher than the controls. MMP and ATP content did not show any significant difference with controls in none of the experimental groups. NNC inhibits the CatSper and reduces human sperm motility and viability. These harmful NNC effects were not due to their impact on MMP or ATP production but are likely because of intracellular calcium reduction and higher ROS production. Progesterone at $10 \mu\text{M}$ concentration had no significant effect and may not be a considerable stimulator for CatSper.

1. Introduction

dSperm motility is one of the important requirements for successful fertilization [1]. Under certain conditions, the sperm becomes hyperactive, and the symmetrical progressive motility of human spermatozoa switches to a powerful whip-like flagella motion. Hyperactivation enables the sperm to pass through the uterine tubes, reach the oocyte, pass through its surrounding layers, and fertilize it [2]. Several signaling pathways are activated during the hyperactivation of the sperm. Increasing intracellular calcium concentration is crucial among the events that lead to hyperactivation [3, 4]. Calcium-signaling pathways mediated various physiological aspects of human sperm action [5, 6]. Intracellular calcium

is essential for controlling sperm motility, capacitation, chemotaxis, hyperactivity, and acrosomal reaction [6]. Spermatozoa contain high- and low-voltage-activated calcium channels (LVA), cyclic nucleotide-gated channels (CNG), and transient receptor potential channels (TRPCs) [7]. Among these channels, a calcium channel of mammalian spermatozoa, CatSper (cation channel of sperm), is recognized as a unique calcium channel of the sperm flagellum. CatSper is a pH-sensitive voltage-dependent calcium channel located on the principal piece of the sperm tail [2], which is activated by alkalization [8], progesterone [9, 10] with an EC₅₀ of 7 nM [11], cyclic nucleotides (cAMP and cGMP), and bovine serum albumin [12]. The importance of CatSper in regulating motility, acrosome reaction, and viability of the

human sperm was detected [13]. The CatSper channel can be blocked by a drug called NNC 55-0396 (NNC), and it was proven that 2 μM NNC blocked the calcium current through this channel in the human sperm [10, 13, 14]. Tamburrino et al. [15] assessed the effects of two CatSper inhibitors, NNC 55-0396 and mibefradil, on human sperm motility. They indicated that both drugs significantly decreased the percentage of the sperm with progressive motility and several kinematic parameters but did not affect the percentage of the hyperactivated sperm. The results demonstrated that CatSper channel expression was positively related to the progressive motility of the sperms.

Progesterone, as a major stimulant of CatSper, activates the CatSper by binding to ABHD2 serine hydrolase (α/β hydrolase domain-containing protein 2) [16]. It has been shown that 10 $\mu\text{g}/\text{mL}$ of progesterone transiently increases the intracellular calcium in the human sperm [17–19], which can be inhibited by the calcium channel blockers, NNC 55-0396 [19].

Some researchers believe that progesterone induces sperm hyperactivation [18, 20]. Progesterone may exert its effect by augmentation in proton pump activity and change of the intracellular pH [21], increasing calcium entry into the sperm [22], activating tyrosine kinases at its nanomolar or micromolar concentrations [23, 24], and stimulating ROS production and ROS-activated pathways [10, 25]. It can, therefore, cause sperm hyperactivity, as well as the promotion of acrosomal reaction. Progesterone could increase the calcium influx with the CatSper channels by activating the phosphatidylinositol 3-kinase (PI3K)/AKT pathway in human spermatozoa [26].

However, increased intracellular calcium, high levels of ROS, and ATP deficiency can damage the mitochondria and accelerate the process of apoptosis [25].

It has been shown in previous studies that NNC blocks the CatSper and inhibits calcium increase in response to progesterone. It is unclear whether NNC or progesterone also affects the mitochondria or ATP production. Therefore, the purpose of the current study was to investigate the effect of CatSper inhibition by NNC or its stimulation by progesterone on the sperm function and then finding a proper understanding of how this happens through the mitochondrial membrane potential changes in ATP and ROS production. For these reasons, sperm motility and viability were assessed, and mitochondrial membrane potential, intracellular ATP levels, and intracellular ROS generation were measured to determine other mechanisms of NNC and progesterone effects.

2. Materials and Methods

2.1. Sample Collection and Preparation. Thirty semen samples were obtained from fertile men (aged 20–40 years) referred to Shiraz Fertility Center. This study was conducted based on the World Health Organization (WHO) criteria [27]. The donors did not have any specific illnesses, and they did not take any supplements or medication; none of them were cigarette or alcohol consumers. They refrained from sexual activity 2 to 7 days before the sample collection. The

initial characteristics of the selected semen samples (before washing), such as volume, pH, and appearance properties, are recorded and reported in Table 1.

This study protocol was approved by the local Medical Ethics Committee at Shiraz Medical University (IR.SUMS.-REC. 1397.788). Detailed consent forms were obtained from all of the participants. Samples were washed using Ham's F10, and motile sperms were prepared by the swim-up method. The samples were diluted (sperm concentration: 20×10^6 sperm/mL). Samples were divided into five groups: control group (contained only Ham's F10), solvent or sham group (0.01% ethanol), 2 μM NNC group, 10 μM progesterone group, and progesterone (10 μM)+NNC (2 μM) combination group. The samples were incubated in a sterile incubator at 37°C and 5% CO_2 for 30 minutes.

2.2. Sperm Motility and Viability Assessment. The sperm motility was analyzed using SQA-VTM Automated Sperm Quality Analyser (version 3.1) ($n = 25$). Ten randomly selected fields or at least 200 sperm cells were analyzed, and progressive, nonprogressive, and immotile spermatozoa were determined. The mean progressive motility and total motility (the sum of progressive and nonprogressive motility) of the samples were obtained and statistically analyzed.

Sperm parameters such as average path velocity (VAP, $\mu\text{m}/\text{sec}$), straight-line velocity (VSL, $\mu\text{m}/\text{sec}$), linearity (LIN, %), amplitude of lateral head displacement (ALH, $\mu\text{m}/\text{sec}$), and curvilinear velocity (VCL, $\mu\text{m}/\text{sec}$) were evaluated for all groups.

Sperm viability was assessed by Eosin Y (E6003, Sigma, USA) staining protocol ($n = 25$). Briefly, an equal amount of sample and Eosin Y was thoroughly mixed. Then, 10 μL of this solution was smeared on the glass slide. After 30 seconds, at least 200 cells were evaluated by an optical microscope (CX41, Olympus, Japan), at a magnification of $\times 200$. The viable sperm cells are excluded from staining, while nonviable sperm cells will be stained red. The average percentage of viable sperm cells was obtained and subjected to statistical analysis.

2.3. Sperm ROS Assessment. The amount of basal and stimulated ROS generation was measured by chemiluminescence assay ($n = 18$). At first, 300 μL of the treated sperm sample was placed in 96-well plates. Then, a dissolved luminol probe (A8511, Sigma, USA) in DMSO (the final concentration of 250 μM) and 12 unit/mL HRP (P6782, Sigma, USA) were added to each sample. The chemiluminescence signal was examined at 37°C for 30 minutes in the experimental groups using a microplate analyzer (Synergy™ HT, BioTek Instruments, USA). The blank wells contained PBS, luminol, and HRP.

2.4. Mitochondrial Membrane Potential Assessment. Mitochondrial membrane potential (MMP) was evaluated using JC1 staining and flow cytometry ($n = 5$). Briefly, one milliliter of the sperm (one million/mL) was mixed with 1 mL of warm PBS buffer. Ten microliters of the prepared JC1 solution (200 μM) (T4069, Sigma, USA) was added to the samples and then incubated for 15 to 30 minutes at 37°C in

TABLE 1: Selected semen sample characteristics before washing.

Semen characteristics	Mean \pm SEM ($n = 30$)
Semen volume (mL)	3.3 \pm 0.2
pH	7.5 \pm 0.2
Sperm concentration ($\times 10^6$ /mL)	84.6 \pm 5.7
Total sperm count ($\times 10^6$)	285 \pm 21.2
Progressive motility (%)	42.1 \pm 0.9
Nonprogressive motility (%)	12.1 \pm 0.3
Immotile sperms (%)	44.8 \pm 0.8
Abnormal sperm morphology (%)	60.9 \pm 1.2
Viability (%)	55.6 \pm 2.2
Viscosity	Normal

Statistical data are represented as mean \pm SEM.

darkness. Then, 50,000 sperms were analyzed using a BD FACSCalibur™ flow cytometer (BD Biosciences, USA) and FlowJo (version 10.4.1) software. As a positive control, 50 μ mol CCCP was added to the samples to destroy the cells and the mitochondrial membrane potential. Excitation was at 488 nm, and emission was at 530 nm and 585 nm. The change of fluorescence light from green (\sim 529 nm) to red (\sim 590 nm) is dependent on the mitochondrial membrane potential; changing the light from green to red means increasing the mitochondrial membrane potential.

2.5. Intracellular ATP Assessment. A bioluminescence assay was performed to assess intracellular ATP ($n = 15$). Twenty-five microliters of the sperm samples was added to a boiling 9-fold volume solution containing 100 mM Tris-HCl at pH 7.75 and 4 mM EDTA. After boiling the sample for 2 minutes at 100°C, we centrifuged the solution at 1000g for 60 seconds. Fifty microliters of the supernatant was removed and stored at -20°C until assay. At the assessment time, 50 μ L of the samples was filled into 96-well plates, 50 μ L of luciferase solution was added, and the data were recorded by a microplate reader with a one-second delay for 10 seconds. In addition, standard concentrations of ATP were prepared according to the kit instructions (11699695001, Roche Holding AG, Switzerland) from the 10^{-10} to 10^{-4} nM concentration, and the data were used to draw the standard curve, which was plotted logarithmically, and the line formula was determined by Excel software. Finally, the concentration was expressed as nanomoles per million sperm.

2.6. Statistical Analysis. At first, the normal distribution of the data was measured by the Shapiro-Wilk normality test. The normally distributed data were analyzed using ANOVA and Tukey's post hoc test. Other data with nonnormal distribution were analyzed by the nonparametric Kruskal-Wallis test. Data were expressed as mean \pm SEM, and $P \leq 0.05$ was considered the statistical significance level. SPSS software (version 24) was used for data analysis.

3. Results

3.1. Effect of Progesterone and NNC on Sperm Motility and Viability. CatSper channel inhibition by NNC reduced the sperm progressive and nonprogressive motility ($P \leq 0.001$) in comparison with controls. Progesterone did not improve the percentage of progressively motile sperm cells, and its combination with NNC did not prevent the harmful effects of NNC (Figure 1).

Progesterone also had no significant effect on the sperm kinematics. NNC significantly reduced ALH and VCL compared to both control and progesterone groups ($P \leq 0.001$); however, the inhibitory effect of NNC did not decrease VSL, LIN, and VAP (Table 2). The combination of progesterone with NNC significantly decreased VCL and ALH compared to the progesterone group ($P \leq 0.001$).

The progesterone group did not show any significant changes in the percentage of viable sperm cells compared to the control group (Figure 2). CatSper channel inhibition by NNC decreased the sperm viability, and progesterone could not prevent this harmful effect of NNC.

3.2. Effect of Progesterone and NNC on Sperm ROS Generation. Recording RLU for 30 minutes and calculating per one million live cells showed a significant increase in ROS production in the NNC and NNC+progesterone groups compared to the control and progesterone-containing groups ($P \leq 0.001$). Meanwhile, ROS production did not show any significant changes in the progesterone group (Table 3).

3.3. Effect of Progesterone and NNC on Sperm MMP. Representative flow cytometry results of JC1 staining and calculated JC-1 red/green fluorescence ratio are shown in Figure 3. Evaluation of the JC-1 red/green fluorescence ratio in all experimental groups showed that progesterone could not increase JC-1 red/green fluorescence ratio compared to the controls. The JC-1 red/green fluorescence ratio showed a slight reduction in the NNC and NNC+progesterone groups; however, this decrease was not statistically significant (Figure 3).

3.4. Effect of Progesterone and NNC on Intracellular ATP. The content of intracellular ATP in the sperm cells was evaluated by the bioluminescence method, and the ATP concentration (nanomole/one million sperm) was calculated. None of the treatments could significantly change the ATP content of the sperm cells (Figure 4).

4. Discussion

The results obtained in the present study showed that incubation of human spermatozoa for 30 minutes in a medium containing 10 μ M progesterone did not significantly change the percentage of viable sperm, as well as sperm progressive and nonprogressive motility. Sperm kinematic evaluation also showed that this progesterone concentration did not considerably change these parameters.

Many studies were done on the effect of progesterone on the sperm. However, the specific conditions of the

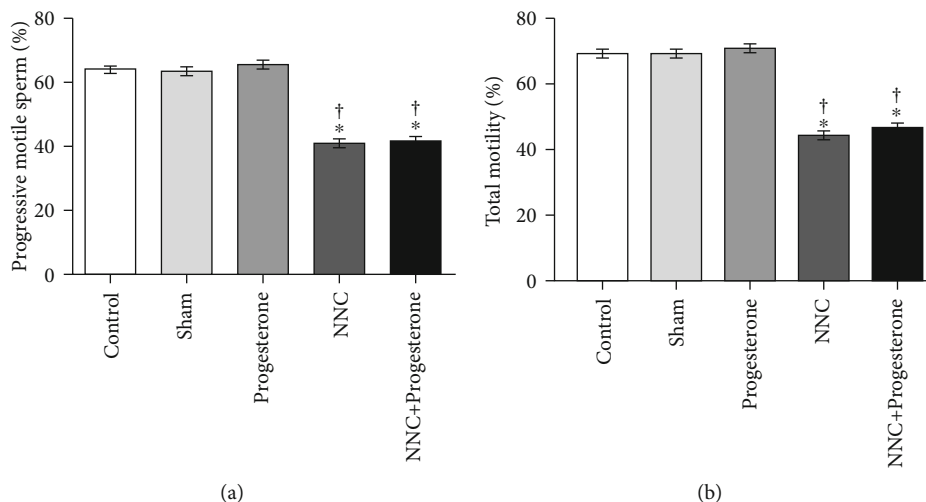


FIGURE 1: Effects of 10 μM progesterone and CatSper channel inhibitor (2 μM NNC) on sperm progressive motility (a) and total motility (b) ($n = 25$). Statistical data are represented as mean \pm SEM. *Significant difference with the control group. †Significant difference with the progesterone group ($P < 0.001$).

TABLE 2: Effects of progesterone and CatSper channel inhibitor (NNC) on sperm kinematics.

Groups ($n = 25$)	VCL ($\mu\text{m}/\text{sec}$)	VSL ($\mu\text{m}/\text{sec}$)	VAP ($\mu\text{m}/\text{sec}$)	LIN (%)	ALH ($\mu\text{m}/\text{sec}$)
Control (Ham's F10)	165.93 \pm 19.72	110.11 \pm 25	116.48 \pm 26	0.66 \pm 0.07	1.99 \pm 0.06
Sham	166.86 \pm 4.97	116.60 \pm 6.25	122.33 \pm 6.23	0.67 \pm 0.017	1.93 \pm 0.05
Progesterone (10 μM)	172.82 \pm 21.82	107.58 \pm 19	113.32 \pm 19	0.62 \pm 0.09	2.19 \pm 0.06
NNC (2 μM)	135.13 \pm 16.2 ^{**†}	92.96 \pm 19	97.19 \pm 18 [*]	0.66 \pm 0.08	1.67 \pm 0.04 ^{*†}
NNC+progesterone	144.02 \pm 28 ^{*†}	95.68 \pm 25	100.20 \pm 26	0.64 \pm 0.08	1.79 \pm 0.06 [†]

Statistical data are represented as mean \pm SEM. *Significant difference with the control group ($P \leq 0.01$). **Significant difference with the control group ($P \leq 0.001$). †Significant difference with the progesterone group ($P < 0.001$).

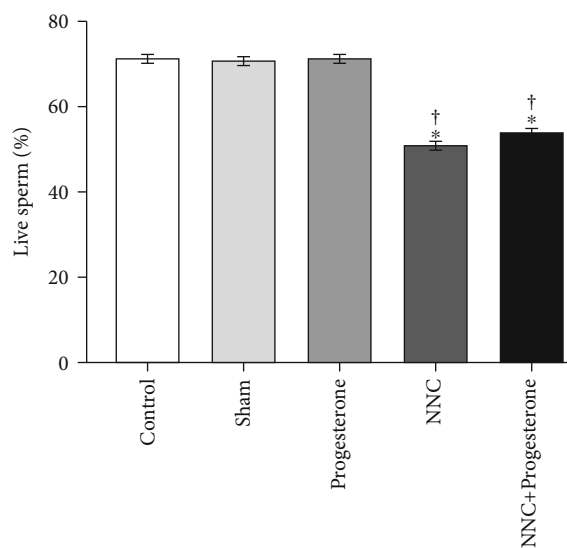


FIGURE 2: Effects of 10 μM progesterone and CatSper channel inhibitor (2 μM NNC) on sperm viability ($n = 25$). Statistical data are represented as mean \pm SEM. *Significant difference with the control group. †Significant difference with the progesterone groups ($P < 0.001$).

TABLE 3: Effects of progesterone and CatSper channel inhibitor (NNC) on sperm ROS generation.

Groups ($n = 18$)	Relative light unit (RLU)	Relative light unit (RLU) per 10^6 live cells
Control (Ham's F10)	400.2 \pm 147.0	91.8 \pm 7.2
Sham	495.50 \pm 39.48	116.7 \pm 15.8
Progesterone (10 μM)	504.5 \pm 289.5	103.7 \pm 8.7
NNC (2 μM)	444.1 \pm 158.0	148.2 \pm 7.5 ^{*†}
NNC+progesterone	450.4 \pm 110.0	156.8 \pm 13.7 ^{*†}

Statistical data are represented as mean \pm SEM. *Significant difference with the control group ($P \leq 0.01$). †Significant difference with the progesterone group ($P < 0.01$).

experiments, such as the concentration of progesterone, duration of the test, and difference in animal species, could influence the results of an experiment. One micrometer of progesterone has no effect on human sperm motility [10, 14]. Moreover, no significant differences were observed between the control group and samples treated with progesterone for cryosurvival rate, motility parameters, or membrane integrity [28].

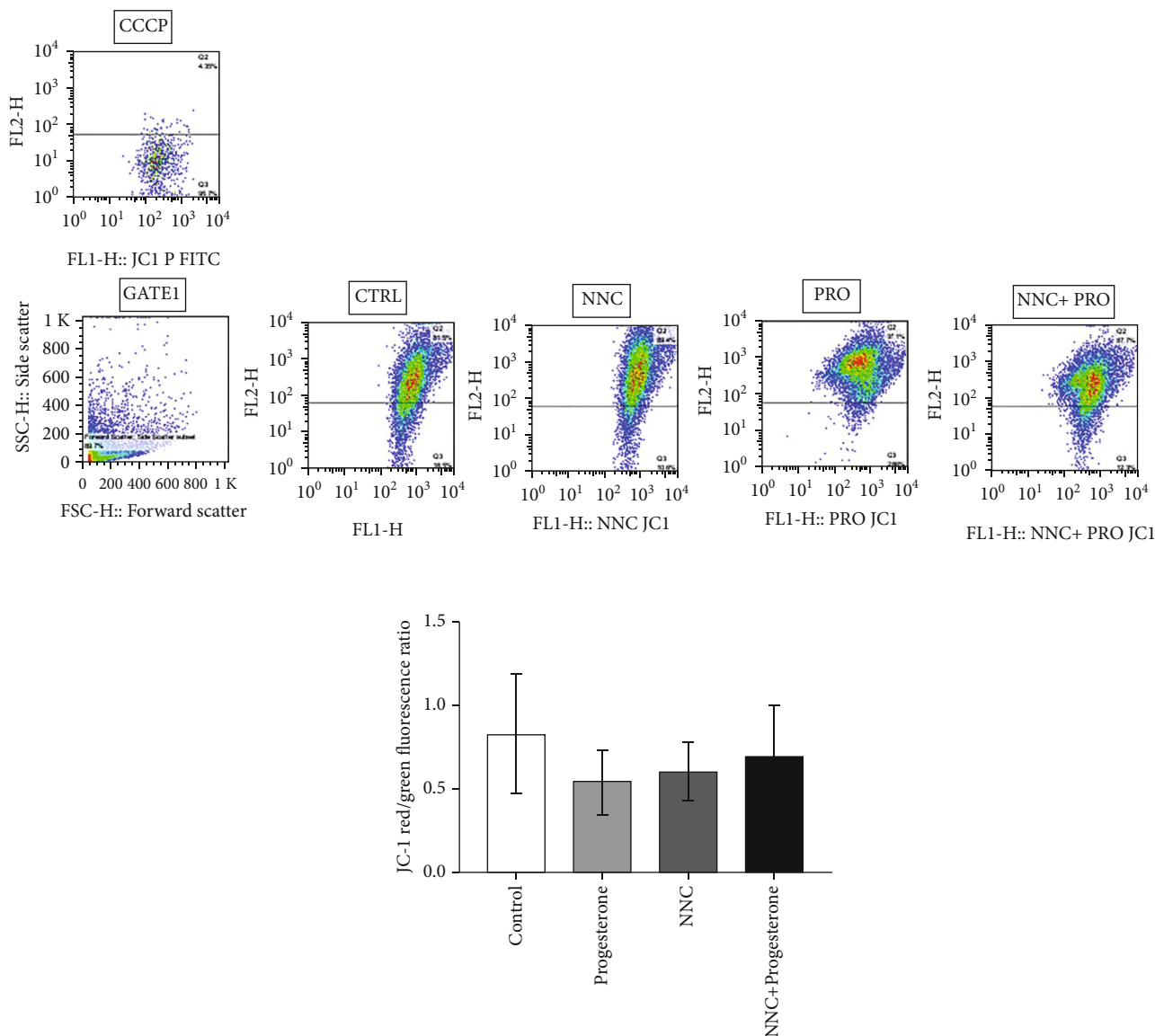


FIGURE 3: Effects of 10 μM progesterone and CatSper channel inhibitor (2 μM NNC) on mitochondrial membrane potential (MMP) ($n = 5$). The pattern of staining with JC1 to differentiate cells in terms of their mitochondrial membrane potential status in the experimental groups. The histogram shows the JC-1 red/green fluorescence ratio. Statistical data are represented as mean \pm SEM.

On the other hand, some studies have shown that progesterone has positive effects on sperm motility depending on concentration and activates major signaling pathways [26, 29]. It has been shown that 10 μM progesterone stimulates the long-distance migration of ram spermatozoa [30]. The oviductal epithelium contains glycans which act as sperm receptors. Sperm was attached to these specific glycans, forming a reservoir of sperm at the oviduct. Progesterone (80 nM) induced sperm release from oviduct glycans within 30 min [31].

Progesterone effects on the sperm may involve cAMP, PKA, L-type and T-type calcium channels, TRPV1, inositol trisphosphate, MAPK [15, 30, 32], and the activation of CatSper by binding to ABHD2 [16]. However, the effects of progesterone on the sperm are temporary and rapid. Endogenous cannabinoids inhibit the CatSper channel, and

progesterone hydrolyzes the inhibitory cannabinoids by binding and activating the highly expressed type II domain dehydrogenase ABHD2 receptor on human spermatozoa [11, 16, 33]. Progesterone increased mRNA and protein levels of ABHD2 in healthy spermatozoa [34]. Previous studies revealed that the role of calcium channels in the sperm progesterone-mediated motility, using calcium channel blockers such as mibefradil, could not completely prevent the progesterone effects. It was suggested that progesterone not only affects calcium entry through calcium channels but also stimulates other signaling mechanisms [12]. Sagare-Patil et al. [26] indicated that lower progesterone levels could partially play a role in sperm motility and tyrosine kinase activation. It takes higher concentrations to induce hyperactivation and acrosome reaction due to activating multiple kinase pathways, including MAPK and AKT.

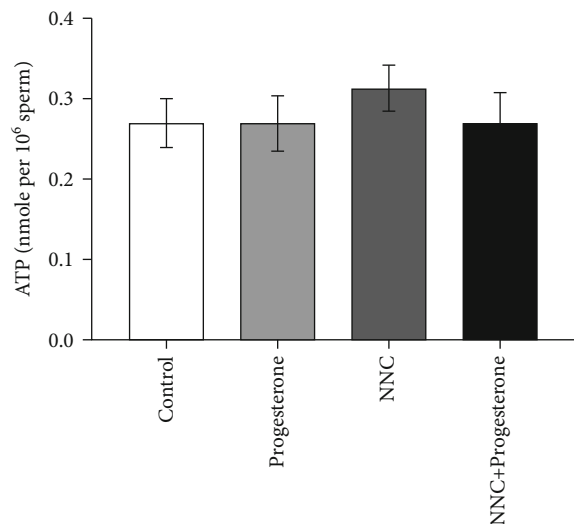


FIGURE 4: Effects of 10 μM progesterone and CatSper channel inhibitor (2 μM NNC) on intracellular ATP levels ($n = 15$). Statistical data are represented as mean \pm SEM.

Sperm VCL analysis showed that a nonsignificant increase in VCL occurred in the progesterone-containing group. Typically, VCL levels above 150 $\mu\text{m}/\text{sec}$ and LIN below 50% are considered as sperm hyperactivity, and the results of the current study showed that progesterone increased the average VCL by about 170 $\mu\text{m}/\text{sec}$. Although the change in VCL of the control and progesterone groups was not statistically significant, this difference is physiologically important and indicates the stimulatory effect of progesterone on the human spermatozoa. Gimeno-Martos et al. [35] showed that progesterone or estrogen receptor agonists could promote the acrosome reaction in ram spermatozoa and increase VCL.

The present study showed that 2 μM NNC significantly reduced the sperm progressive and nonprogressive motility and the percentage of living spermatozoa. Our results and other reports emphasize the importance of the CatSper calcium channel function on human sperm motility and viability [15, 18].

However, since the addition of NNC to the spermatozoon medium could not immobilize all motile spermatozoa, it can be concluded that NNC does not completely inhibit the calcium entry. Previous studies showed that other calcium channels such as low-voltage-activated (LVA), cyclic nucleotide-gated (CNG), and transient receptor potential canonical (TRPC) channels besides CatSper play an essential role in sperm physiology and are not inhibited by NNC [22, 23]. In addition to calcium, other factors, including cAMP, tyrosine phosphorylation, and intracellular alkalinity, are also involved in controlling the sperm motility [36, 37].

In fact, progesterone-induced increased intracellular calcium was significantly reduced by CatSper inhibitors [17, 19]. However, the question of whether CatSper channels are involved in the multiple effects of progesterone on the sperm function [38] has not been elucidated yet. NNC incubation reduced the motility of boar spermatozoa, and this decrease was not compensated even by adding

10 μM progesterone [39]. In our experiment, NNC was added to the medium after progesterone; however, the effect of NNC was dominant, and progesterone could not prevent NNC-reducing effects on the sperm viability and motility.

Our finding revealed that progesterone causes a slight increase in sperm ROS production, which was not statistically significant. In another study on human spermatozoa, progesterone via calcium entry has been implicated in ROS production by the NOX5 enzyme [10].

Progesterone induces oxidative stress through its effect on nicotinamide adenine dinucleotide (NAD) and reduced superoxide dismutase (SOD) gene expression of the hepatocytes and endothelial cells [10, 40]. However, other studies on boar spermatozoa, endometrial cancer cells, and female rat cardiomyocytes showed that progesterone had no significant effect on ROS production [41–43].

In the present study, preliminary analysis of ROS production showed that NNC had no effect on ROS production, but as mentioned previously, the addition of NNC to spermatozoa containing medium caused a considerable mortality rate. When ROS production was calculated by one million living cells, it was found that NNC significantly increased the ROS production by living spermatozoa. It has also been shown that NNC can inhibit P450 enzyme activity, which in turn increases apoptosis [44].

Mitochondria (via the NAD-dependent redox reaction) and sperm plasma membrane (via NADPH oxidase system, especially NOX5) are two major sites of ROS generation. Increased intracellular calcium plays a major role in the ROS production process at both sites [45].

In the present study, intracellular calcium was not measured, but we showed in our previous study that intracellular calcium was reduced by NNC [10]. Due to the inhibition of the CatSper calcium channel by NNC, the decrease in intracellular calcium in the presence of this substance seems reasonable. Therefore, increased ROS production by NNC could not be related to intracellular calcium levels. NNC is likely to increase the ROS generation by affecting antioxidants and reducing their activity. Finding the exact mechanisms requires further studies.

In the present study, progesterone did not affect intracellular ATP levels. The presence of progesterone could not increase the MMP levels. The results of two studies on boar spermatozoa also partially confirmed the present study findings regarding MMP and ATP [42, 46].

Furthermore, NNC nonsignificantly decreased MMP compared to the controls. Although intracellular calcium levels were not determined in this study, it is reasonable to assume that treatment with NNC reduced the basal calcium entry and consequently decreased the mitochondrial MMP. It was demonstrated that the increase in ROS could lead to a decrease in the MMP.

5. Conclusion

The results of this study showed that the activity of the CatSper calcium channel could play an essential role in the sperm physiology, including its motility and viability. The stimulatory effects of progesterone on the sperm are likely

to be very rapid and transient, and transient effects of progesterone were not observable at this measurement time. Another possibility is that in human sperm, progesterone is not a stimulator for CatSper. It is also possible that sperm must undergo capacitation before adding progesterone to the culture media. In human spermatozoa, the role of neither CatSper nor progesterone has been fully elucidated. The mechanism of the effect of progesterone is still unknown and needs further research. NNC ($2\ \mu\text{M}$ concentration) completely blocked the CatSper channel and reduced the sperm motility and kinematic parameters. NNC also increased the sperm mortality rate. These effects of NNC were not due to an effect on mitochondrial membrane potential or ATP production, but the adverse impacts of NNC are likely to be due to increased ROS production in addition to reduced calcium entry. Progesterone could not prevent the harmful effects of NNC on the human sperm.

Data Availability

The dataset on which this paper is based is too large to be retained or publicly archived with available resources. Documentation and methods used to support this study are available by emailing the corresponding author.

Disclosure

This manuscript was extracted from the MSc thesis of Setareh Maleki.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

The main project contributors SK and NK participated effectively in designing and managing the project. SM, BE, HMA, and FM collaborated in data collection and statistical analyses. All authors collaborated effectively in data interpretation, manuscript drafting, and copyediting.

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References

- [1] R. Dcunha, R. S. Hussein, H. Ananda et al., "Current insights and latest updates in sperm motility and associated applications in assisted reproduction," *Reproductive Sciences*, vol. 29, no. 1, pp. 7–25, 2022.
- [2] P. V. Lishko and N. Mannowetz, "CatSper: a unique calcium channel of the sperm flagellum," *Current Opinion in Physiology*, vol. 2, pp. 109–113, 2018.
- [3] W. Alasmari, C. L. Barratt, S. J. Publicover et al., "The clinical significance of calcium-signalling pathways mediating human sperm hyperactivation," *Human Reproduction*, vol. 28, no. 4, pp. 866–876, 2013.
- [4] D. Ren and J. Xia, "Calcium signaling through CatSper channels in mammalian fertilization," *Physiology*, vol. 25, no. 3, pp. 165–175, 2010.
- [5] E. Mata-Martínez, C. Sánchez-Cárdenas, J. C. Chávez et al., "Role of calcium oscillations in sperm physiology," *Biosystems*, vol. 209, article 104524, 2021.
- [6] K. Yoshida, K. Shiba, A. Sakamoto et al., " Ca^{2+} efflux via plasma membrane Ca^{2+} -ATPase mediates chemotaxis in ascidian sperm," *Scientific Reports*, vol. 8, no. 1, article 16622, 2018.
- [7] A. Darszon, T. Nishigaki, C. Beltran, and C. L. Trevino, "Calcium channels in the development, maturation, and function of spermatozoa," *Physiological Reviews*, vol. 91, no. 4, pp. 1305–1355, 2011.
- [8] J. Y. Hwang and J.-J. Chung, "CATSPER calcium channels: twenty years on," *Physiology*, vol. 38, no. 3, pp. 125–140, 2022.
- [9] P. V. Lishko, Y. Kirichok, D. Ren, B. Navarro, J. J. Chung, and D. E. Clapham, "The control of male fertility by spermatozoan ion channels," *Annual Review of Physiology*, vol. 74, no. 1, pp. 453–475, 2012.
- [10] H. Ghanbari, S. Keshtgar, H. R. Zare, and B. Ghahesi-Fard, "Inhibition of CatSper and Hv1 channels and NOX5 enzyme affect progesterone-induced increase of intracellular calcium concentration and ROS generation in human sperm," *Iranian Journal of Medical Sciences*, vol. 44, no. 2, pp. 127–134, 2019.
- [11] N. Mannowetz, M. R. Miller, and P. V. Lishko, "Regulation of the sperm calcium channel CatSper by endogenous steroids and plant triterpenoids," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 114, no. 22, pp. 5743–5748, 2017.
- [12] X. H. Sun, Y. Y. Zhu, L. Wang et al., "The Catsper channel and its roles in male fertility: a systematic review," *Reproductive Biology and Endocrinology*, vol. 15, no. 1, p. 65, 2017.
- [13] H. Ghanbari, S. Keshtgar, and M. Kazeroni, "Inhibition of the CatSper channel and NOX5 enzyme activity affects the functions of the progesterone-stimulated human sperm," *Iranian Journal of Medical Sciences*, vol. 43, no. 1, pp. 18–25, 2018.
- [14] S. Keshtgar, H. Ghanbari, E. Ghani, and S. M. Shid Moosavi, "Effect of CatSper and Hv1 channel inhibition on progesterone stimulated human sperm," *Journal of Reproduction & Infertility*, vol. 19, no. 3, pp. 133–139, 2018.
- [15] L. Tamburrino, S. Marchiani, F. Minetti, G. Forti, M. Muratori, and E. Baldi, "The CatSper calcium channel in human sperm: relation with motility and involvement in progesterone-induced acrosome reaction," *Human Reproduction*, vol. 29, no. 3, pp. 418–428, 2014.
- [16] M. R. Miller, N. Mannowetz, A. T. Iavarone et al., "Unconventional endocannabinoid signaling governs sperm activation via the sex hormone progesterone," *Science*, vol. 352, no. 6285, pp. 555–559, 2016.
- [17] P. V. Lishko, I. L. Botchkina, and Y. Kirichok, "Progesterone activates the principal Ca^{2+} channel of human sperm," *Nature*, vol. 471, no. 7338, pp. 387–391, 2011.

- [18] J. F. Smith, O. Syritsyna, M. Fellous et al., "Disruption of the principal, progesterone-activated sperm Ca^{2+} channel in a CatSper2-deficient infertile patient," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 17, pp. 6823–6828, 2013.
- [19] T. Strünker, N. Goodwin, C. Brenker et al., "The CatSper channel mediates progesterone-induced Ca^{2+} influx in human sperm," *Nature*, vol. 471, no. 7338, pp. 382–386, 2011.
- [20] V. Sagare-Patil, R. Bhilawadikar, M. Galvankar, K. Zaveri, I. Hinduja, and D. Modi, "Progesterone requires heat shock protein 90 (HSP90) in human sperm to regulate motility and acrosome reaction," *Journal of Assisted Reproduction and Genetics*, vol. 34, no. 4, pp. 495–503, 2017.
- [21] A. Grimm, E. E. Biliouris, U. E. Lang, J. Gotz, A. G. Mensah-Nyagan, and A. Eckert, "Sex hormone-related neurosteroids differentially rescue bioenergetic deficits induced by amyloid- β or hyperphosphorylated tau protein," *Cellular and Molecular Life Sciences*, vol. 73, no. 1, pp. 201–215, 2016.
- [22] Y. Kirichok and P. V. Lishko, "Rediscovering sperm ion channels with the patch-clamp technique," *Molecular Human Reproduction*, vol. 17, no. 8, pp. 478–499, 2011.
- [23] A. S. Lopez-Torres and M. Chirinos, "Modulation of human sperm capacitation by progesterone, estradiol, and luteinizing hormone," *Reproductive Sciences*, vol. 24, no. 2, pp. 193–201, 2017.
- [24] L. Tamburrino, S. Marchiani, M. Muratori, M. Luconi, and E. Baldi, "Progesterone, spermatozoa and reproduction: an updated review," *Molecular and Cellular Endocrinology*, vol. 516, article 110952, 2020.
- [25] A. Amaral, B. Lourenco, M. Marques, and J. Ramalho-Santos, "Mitochondria functionality and sperm quality," *Reproduction*, vol. 146, no. 5, pp. R163–R174, 2013.
- [26] V. Sagare-Patil, M. Vernekar, M. Galvankar, and D. Modi, "Progesterone utilizes the PI3K-AKT pathway in human spermatozoa to regulate motility and hyperactivation but not acrosome reaction," *Molecular and Cellular Endocrinology*, vol. 374, no. 1–2, pp. 82–91, 2013.
- [27] WHO, *WHO Laboratory Manual for the Examination and Processing of Human Semen*, World Health Organization, Geneva, 5th edition, 2010, <https://apps.who.int/iris/handle/10665/44261>.
- [28] N. K. Duru, M. Morshedi, A. Schuffner, and S. Oehninger, "Semen treatment with progesterone and/or acetyl-L-carnitine does not improve sperm motility or membrane damage after cryopreservation-thawing," *Fertility and Sterility*, vol. 74, no. 4, pp. 715–720, 2000.
- [29] V. Sagare-Patil, M. Galvankar, M. Satiya, B. Bhandari, S. K. Gupta, and D. Modi, "Differential concentration and time dependent effects of progesterone on kinase activity, hyperactivation and acrosome reaction in human spermatozoa," *International Journal of Andrology*, vol. 35, no. 5, pp. 633–644, 2012.
- [30] C. Martinez-Rodriguez, L. Anel-Lopez, M. Alvarez et al., "Progesterone stimulates the long-distance migration of capacitated ram spermatozoa through viscous media under geotactic condition," *Theriogenology*, vol. 118, pp. 7–15, 2018.
- [31] M. Sharif, V. Hickl, G. Juarez et al., "Hyperactivation is sufficient to release porcine sperm from immobilized oviduct glycans," *Scientific Reports*, vol. 12, no. 1, p. 6446, 2022.
- [32] A. Mahajan, P. Sharma, A. K. Mishra et al., "Interplay mechanisms between progesterone and endocannabinoid receptors in regulating bull sperm capacitation and acrosome reaction," *Journal of Cellular Physiology*, vol. 237, no. 7, pp. 2888–2912, 2022.
- [33] M. P. Baggelaar, H. den Dulk, B. I. Florea et al., "ABHD2 inhibitor identified by activity-based protein profiling reduces acrosome reaction," *ACS Chemical Biology*, vol. 14, no. 12, p. 2943, 2019.
- [34] F. Jiang, Y. Zhu, Y. Chen et al., "Progesterone activates the cyclic AMP-protein kinase A signalling pathway by upregulating ABHD2 in fertile men," *The Journal of International Medical Research*, vol. 49, no. 3, article 300060521999527, 2021.
- [35] S. Gimeno-Martos, M. Santorromán-Nuez, J. A. Cebrián-Pérez, T. Muiño-Blanco, R. Pérez-Pé, and A. Casao, "Involvement of progesterone and estrogen receptors in the ram sperm acrosome reaction," *Domestic Animal Endocrinology*, vol. 74, article 106527, 2021.
- [36] M. G. Buffone, E. V. Wertheimer, P. E. Visconti, and D. Krapf, "Central role of soluble adenylyl cyclase and cAMP in sperm physiology," vol. 1842, no. 12 Part B, pp. 2610–2620, 2014.
- [37] J. Zhou, L. Chen, J. Li et al., "The semen pH affects sperm motility and capacitation," *PLoS One*, vol. 10, no. 7, pp. e0132974–e0132974, 2015.
- [38] E. Baldi, M. Luconi, C. Krausz, and G. Forti, "Editorial commentary: Progesterone and spermatozoa: a long-lasting liaison comes to definition," *Human Reproduction*, vol. 26, no. 11, pp. 2933–2934, 2011.
- [39] A. Vicente-Carrillo, M. Alvarez-Rodriguez, and H. Rodriguez-Martinez, "The CatSper channel modulates boar sperm motility during capacitation," *Reproductive Biology*, vol. 17, no. 1, pp. 69–78, 2017.
- [40] T. A. Fedotcheva, A. G. Kruglov, V. V. Teplova, N. I. Fedotcheva, V. M. Rzhizhnikov, and N. L. Shimanovskii, "Effect of steroid hormones on production of reactive oxygen species in mitochondria," *Biofizika*, vol. 57, no. 6, pp. 792–795, 2012.
- [41] H. Nguyen and V. Syed, "Progesterone inhibits growth and induces apoptosis in cancer cells through modulation of reactive oxygen species," *Gynecological Endocrinology*, vol. 27, no. 10, pp. 830–836, 2011.
- [42] L. Ramio-Lluch, J. M. Fernandez-Novell, A. Pena et al., "In vitro' capacitation and acrosome reaction are concomitant with specific changes in mitochondrial activity in boar sperm: evidence for a nucleated mitochondrial activation and for the existence of a capacitation-sensitive subpopulational structure," *Reproduction in Domestic Animals*, vol. 46, no. 4, pp. 664–673, 2011.
- [43] C. Rattanasopa, S. Phungphong, J. Wattanapernpool, and T. Bupha-Intr, "Significant role of estrogen in maintaining cardiac mitochondrial functions," *The Journal of Steroid Biochemistry and Molecular Biology*, vol. 147, pp. 1–9, 2015.
- [44] A. Quesada, P. H. Bui, G. E. Homanics, O. Hankinson, and A. Handforth, "Comparison of mibefradil and derivative NNC 55-0396 effects on behavior, cytochrome P450 activity, and tremor in mouse models of essential tremor," *European Journal of Pharmacology*, vol. 659, no. 1, pp. 30–36, 2011.
- [45] S. Dutta, A. Majzoub, and A. Agarwal, "Oxidative stress and sperm function: a systematic review on evaluation and management," *Arab Journal of Urology*, vol. 17, no. 2, pp. 87–97, 2019.
- [46] M. Yeste, J. M. Fernandez-Novell, L. Ramio-Lluch et al., "Intracellular calcium movements of boar spermatozoa during 'in vitro' capacitation and subsequent acrosome exocytosis follow a multiple-storage place, extracellular calcium-dependent model," *Andrology*, vol. 3, no. 4, pp. 729–747, 2015.