Research Article

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

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1. Introduction

Sperm 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 mibebradil, 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 μg/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 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, μm/sec), straight-line velocity (VSL, μm/sec), linearity (LIN, %), amplitude of lateral head displacement (ALH, μm/sec), and curvilinear velocity (VCL, μm/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 ×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. 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 (spem concentration: 20 × 10⁶ 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% CO2 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.

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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
darkness. Then, 50,000 sperms were analyzed using a BD FACS Calibur™ flow cytometer (BD Biosciences, USA) and FlowJo (version 10.4.1) software. As a positive control, 50 μmol 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 (~529 nm) to red (~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 1000 g for 60 seconds. Fifty microliters of the supernatant was added to the controls. The JC-1 red/green fluorescence ratio showed changes in the percentage of viable sperm cells, 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 ± 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 VSL 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 μM 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

<table>
<thead>
<tr>
<th>Table 1: Selected semen sample characteristics before washing.</th>
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<tbody>
<tr>
<td>Semen characteristics</td>
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<td>------------------------</td>
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<tr>
<td>Semen volume (mL)</td>
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<tr>
<td>pH</td>
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<tr>
<td>Sperm concentration ((\times 10^6)/mL)</td>
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<tr>
<td>Total sperm count ((\times 10^6))</td>
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<td>Progressive motility (%)</td>
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<tr>
<td>Nonprogressive motility (%)</td>
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<tr>
<td>Immotile sperms (%)</td>
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<tr>
<td>Abnormal sperm morphology (%)</td>
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<td>Viability (%)</td>
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<td>Viscosity</td>
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Statistical data are represented as mean ± SEM.
progesterone group (P in inhibitor (2 μM NNC) are represented as † control group. groups (P = 2.500). Statistical data are represented as † control group. 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 ± 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.

<table>
<thead>
<tr>
<th>Groups (n = 25)</th>
<th>VCL (μm/sec)</th>
<th>VSL (μm/sec)</th>
<th>VAP (μm/sec)</th>
<th>LIN (%)</th>
<th>ALH (μm/sec)</th>
</tr>
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<tbody>
<tr>
<td>Control (Ham’s F10)</td>
<td>165.93 ± 19.72</td>
<td>110.11 ± 25</td>
<td>116.48 ± 26</td>
<td>0.66 ± 0.07</td>
<td>1.99 ± 0.06</td>
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<tr>
<td>Sham</td>
<td>166.86 ± 4.97</td>
<td>116.60 ± 6.25</td>
<td>122.33 ± 6.23</td>
<td>0.67 ± 0.017</td>
<td>1.93 ± 0.05</td>
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<tr>
<td>Progesterone (10 μM)</td>
<td>172.82 ± 21.82</td>
<td>107.58 ± 19</td>
<td>113.32 ± 19</td>
<td>0.62 ± 0.09</td>
<td>2.19 ± 0.06</td>
</tr>
<tr>
<td>NNC (2 μM)</td>
<td>135.13 ± 16.2**†</td>
<td>92.96 ± 19</td>
<td>97.19 ± 18*</td>
<td>0.66 ± 0.08</td>
<td>1.67 ± 0.04†</td>
</tr>
<tr>
<td>NNC+progesterone</td>
<td>144.02 ± 28**†</td>
<td>95.68 ± 25</td>
<td>100.20 ± 26</td>
<td>0.64 ± 0.08</td>
<td>1.79 ± 0.06†</td>
</tr>
</tbody>
</table>

Statistical data are represented as mean ± SEM. *Significant difference with the control group (P ≤ 0.01). **Significant difference with the control group (P ≤ 0.001). †Significant difference with the progesterone group (P < 0.001).

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

<table>
<thead>
<tr>
<th>Groups (n = 18)</th>
<th>Relative light unit (RLU) per 10^6 live cells</th>
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</thead>
<tbody>
<tr>
<td>Control (Ham’s F10)</td>
<td>400.2 ± 147.0</td>
</tr>
<tr>
<td>Sham</td>
<td>495.50 ± 39.48</td>
</tr>
<tr>
<td>Progesterone (10 μM)</td>
<td>504.5 ± 289.5</td>
</tr>
<tr>
<td>NNC (2 μM)</td>
<td>444.1 ± 158.0</td>
</tr>
<tr>
<td>NNC+progesterone</td>
<td>450.4 ± 110.0</td>
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</table>

Statistical data are represented as mean ± SEM. *Significant difference with the control group (P ≤ 0.01). †Significant difference with the progesterone group (P < 0.01).

Figure 1: Effects of 10 μM progesterone and CatSper channel inhibitor (2 μM NNC) on sperm viability (n = 25). Statistical data are represented as mean ± SEM. *Significant difference with the control group. †Significant difference with the progesterone groups (P < 0.001).

Figure 2: Effects of 10 μM progesterone and CatSper channel inhibitor (2 μM NNC) on sperm cryosurvival rate, motility parameters, or membrane integrity.

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].
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 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 ± SEM.
Sperm VCL analysis showed that a nonsignificant increase in VCL occurred in the progesterone-containing group. Typically, VCL levels above 150 μm/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 μm/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 spermatozoa 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 either CatSper nor progesterone has been fully elucidated. The mechanism of the effect of progesterone is still unknown and needs further research. NNC (2 μM concentration) completely blocked the CatSper channel and reduced the sperm motility and kinetic 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 were 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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