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Matrine compromises mouse sperm functions by a $[Ca^{2+}]_i$-related mechanism

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ABSTRACT

Matrine, a bioactive alkaloid widely used in Chinese medicine, inhibits mouse sperm functions *in vitro*. In this study, we investigated the reproductive toxicity of matrine to male mice *in vivo*. C57BL/6J mice were administered with daily doses of 0, 1, 10 and 50 mg/kg matrine by intraperitoneal injection for 30 days. The results showed that matrine did not affect testis size, testis weight, sperm count and sperm viability, but it significantly inhibited total motility, progressive motility, linear velocity, capacitation and the progesterone-induced acrosome reaction of mouse sperm. Furthermore, the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), a key regulator of sperm function, was reduced in sperm of matrine-exposed mice. The current and gene expression of the sperm specific Ca^{2+} channel, CatSper, which modulates Ca^{2+} influx in sperm, were decreased in testes of matrine-exposed mice. These results indicate that matrine inhibits mouse sperm functions by a $[Ca^{2+}]_i$-related mechanism via CatSper channel.

1. Introduction

Matrine is the main bioactive component of *Sophora flavescens* (kushen) and *Sophora subprostrata* (shandougen) which are the well-known traditional Chinese medicines used in the treatment of cancer, psoriasis and other diseases [1]. Matrine is a quinolizidine alkaloid with various biological effects, such as antiviral, anti-arrhythmic, anti-fibrotic, anti-inflammatory, anti-tumor and insecticidal activities [1–6]. Therefore, It has high frequency of clinical and agricultural uses in China [7,8]. It is regarded that matrine is relatively safe to humans and animals during its long-term uses in China [8,9]. However, several studies suggested this alkaloid had potential nervous toxicity [10–13]. Additionally, our recent study found that matrine had an acute toxicity to mouse sperm *in vitro* [14]. Thus, further study is required to evaluate the male reproductive toxicity of matrine and explore the underlying toxicity mechanism.

In mammals, sperm are produced from spermatogonia by spermatogenesis in the testes [15]. After ejaculated into the female reproductive tract, sperm undergo a series of physiological and biochemical changes that fulfill several functions, such as motility, capacitation and the acrosome reaction (AR) [16–18]. All these functions are essential for sperm to swim through oviductal mucus and penetrate the oocyte [19–22]. Lack of these functions may cause male infertility [23]. As reported previously, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is pivotal to sperm function [24,25]. The vital source of sperm $[Ca^{2+}]_i$ is Ca^{2+} influx from the extracellular environment mediated by Ca^{2+}-permeable channels [26]. The CatSper channel, a pH-dependent voltage-gated Ca^{2+} channel, mediates the predominant Ca^{2+} current of mouse sperm [27]. It consists of four α subunits, CatSper1–4, which are all required for male fertility [23]. In our recent study, we found that matrine inhibited mouse sperm functions *in vitro* by reducing the sperm $[Ca^{2+}]$i. However, whether matrine has reproductive toxicity to male mice *in vivo* is not clear [14].

In this study, we focused on assessing the effect of matrine on testis factors, sperm count, sperm viability and sperm functions in male mice. To explore the underlying mechanisms, sperm $[Ca^{2+}]_i$, the CatSper current, and *CatSper* gene expression were also examined. Our results will provide novel knowledge to help understand the side-effect and environmental safety of matrine in clinical and agricultural applications.

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2. Materials and methods

2.1. Animals and treatments

Sixty male C57BL/6J mice (20–25 g; 6–8 weeks) were purchased from the Laboratory Animal Center of Nanchang University in Jiangxi Province, China. All animal procedures were conducted in accordance with the recommendations of the National Institutes of Health guidelines and approved by the Animal Care and Use Committee of Nanchang University (Permit Number: SYXK2010-0002). Matrine (CAS-number: 519-02-8; HPLC purity: ≥95%; molecular weight: 248.36; molecular formula: $C_{15}H_{24}N_2O$) was purchased from National Institutes for Food and Drug Control (Beijing, China).

Male mice were randomized into four groups ($n = 15$ mice/group), 0 (control), 1, 10 and 50 mg/kg, by body weight to provide uniform mean body weights across groups. All mice were given ad libitum access to food and water in the condition of temperature of 20–25 °C and under a 12/12 h light/dark schedule and acclimated to the laboratory for 1 week. Matrine was dissolved in phosphate buffer saline (PBS, pH 7.2) solution and administered to mice in 1, 10 and 50 mg/kg groups by intraperitoneal injection at single daily doses of 1, 10 and 50 mg/kg body weight for 30 days. The dose given to mice was based on a recent determination of individual body weight which was weighed every day just before administration. The mice were given 0.2 ml of test solution each day. The mice in control group (0 mg/kg group) received an equivalent volume of PBS for 30 days.

At the termination of the administration, mice were weighed. The testes were taken for photographing, weighing and RNA isolating. Sperm were released from cauda epididymis described previously [28]. The sperm viability was examined by eosin staining according to our previous publication [14]. The sperm count and motility were analyzed in a Computer-Assisted Sperm Analysis (CASA) machine (WLJY-9000, WeiLi. Co., Ltd., Beijing, China) as described [14]. Four parameters (cell density, total motility, progressive motility and linear velocity) were recorded.

2.2. Acrosomal status evaluation

For evaluating capacitation and spontaneous AR, sperm isolated from matrine-exposed mice were capacitated in human tubal fluid (HTF) medium (Merck Millipore Corporation, Darmstadt, Germany) in a 37 °C, 5% CO_2 incubator for 60 min. For evaluating progesterone-induced AR, the capacitated sperm were incubated with 20 μM progesterone for an additional 30 min. Subsequently, capacitation and AR were assessed using chlortetracycline (CTC) staining as previously described [14].

2.3. Sperm $[Ca^{2+}]_i$ assessment

Sperm $[Ca^{2+}]_i$ was monitored using a Ca^{2+} indicator, Fluo-4 AM (Molecular Probes, Waltham, MA, USA). The sperm staining, immobilization and imaging were performed as described in our recent publication [14]. The sperm $[Ca^{2+}]_i$ was indicated as the mean fluorescence intensity of Fluo-4. Totally, 100 sperm from each group were calculated.

2.4. Sperm patch-clamping recording

For recording the CatSper current, sperm were bathed in HS medium (135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 20 mM HEPES, 5 mM glucose, 10 mM lactic acid, and 1 mM Na-pyruvate at pH 7.4 with NaOH). The pipette solution (135 mM CsMes, 5 mM CsCl, 10 mM HEPES, and 10 mM EGTA, pH 7.2) was used. Whole-cell recording was gained through the sperm cytosolic droplet described previously [27]. The monovalent current of CatSper was examined using voltage-clamp ramp protocol from −100 to +100 mV perfused with a sodium based divalent-free (DVF) solution (150 mM NaCl, 20 mM HEPES, and 5 mM EDTA, pH 7.4).

2.5. Real-time PCR analysis

Total RNAs were homogenized and extracted from the testes of the matrine-exposed mice using TRIzol® Reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The RNA was treated with RQ1 RNase-free DNase (Promega Corporation, Madison, WI, USA). First-strand cDNAs were synthesized from 2 μg of total RNAs with oligo $(dT)_{18}$ primer using RevertAid first strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription was performed at 42 °C for 30 min, followed by 30 min at 37 °C. Quantitative real-time PCR was carried out on StepOnePlus Real-Time System (Thermo Fisher Scientific, Waltham, MA, USA). Each 20 μL PCR reaction contained 1 μL of RT product, 10 μL of SYBR® Premix Ex Taq™ mix (TAKARA, Dalian, China), and 0.5 pM of each primer (Supplemental Table S1). The samples were heated to 95 °C for 2 min, followed by 40 cycles of 15 s at 95 °C, 15 s at 58 °C, and 15 s at 72 °C. The $2^{-D\Delta Ct}$ method was used to calculate the relative expression. The transcript levels of examined genes were quantitatively normalized to the transcript level of the housekeeping gene *β-actin*.

2.6. Western blot

Total proteins from the testes of the matrine-exposed mice were prepared using TRIzol® Reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Protein concentration was determined by the BCA method and 50 μg proteins were electrophoresed on SDS-polyacrylamide gels and transferred onto a polyvinylidenedifluoride membrane (GE Healthcare, Fairfield, CT, USA). After incubated with the primary antibodies (Supplemental Table S2) and HRP-conjugated goat anti-rabbit or anti-mouse IgG (Thermo Fisher Scientific, Waltham, MA, USA), the filter was visualized using the ECL detection kit (Thermo Fisher Scientific, Waltham, MA, USA).

2.7. Statistical analysis

Data are expressed as mean ± SEM. Differences between control and different samples were assessed by One-way ANOVA analysis using statistics software GraphPad Prism (version 5.01). Statistically significant difference was determined at $P < 0.05$.

3. Results

3.1. Matrine has no toxicity on testis development and spermatogenesis of male mice

In this study, we focused to examine the male reproductive toxicity of matrine *in vivo*. According to the clinical dose in China (2.5 mg/kg, daily, equivalent to about 28 mg/kg for mouse) [29], we set a range of matrine doses (1, 10 and 50 mg/kg, daily) to examine the reproductive toxicity of matrine to male mice *in vivo*. No mice were dead after matrine exposure for 30 days. The body weight of mice in each groups showed no significant difference (Fig. 1A, $P > 0.05$). Additionally, no significant differences were observed between the controls and male mice exposed to matrine with regard to the testis weights (Fig. 1B, $P > 0.05$), the ratio of testis weights to body weights (Fig. 1C, $P > 0.05$) and testis sizes (Fig. 1D, $P > 0.05$). These results correlated with the previous study that matrine has no developmental toxicity to mice [10]. To further determine whether matrine affects spermatogenesis, the sperm were prepared from cauda epididymides of matrine-exposed mice