Original Paper

Matrine Inhibits Mouse Sperm Function by Reducing Sperm $[Ca^{2+}]_i$ and Phospho-ERK1/2

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Key Words

Matrine • $[Ca^{2+}]_i$ • ERK1/2; • Sperm ion channels • Sperm motility, acrosome reaction

Abstract

Background: Matrine is a bioactive alkaloid that has a variety of pharmacological effects and is widely used in Chinese medicine. However, its effects on male reproduction are not well known. In this study, we aimed to investigate the *in vitro* toxicity of matrine on mature mouse sperm. **Methods:** Mouse cauda epididymal sperm were exposed to matrine (10–200 μ M) *in vitro*. The viability, motility, capacitation, acrosome reaction and fertilization ability of the mouse sperm were examined. Furthermore, the intracellular calcium concentration ($[Ca^{2+}]_i$), calcium (Catsper) and potassium (Ksper) currents, and phosphorylation of extracellular signal regulated kinases 1/2 (p-ERK1/2) of the sperm were analyzed. **Results:** After exposure to 100 μ M or more of matrine, mouse cauda epididymal sperm exhibited a significant reduction in total motility, progressive motility, linear velocity and acrosome reaction rate induced by Ca^{2+} ionophore A23187. As a result, the fertilization ability of mouse sperm was remarkably decreased by matrine. Our data further demonstrated that matrine significantly reduced sperm $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ -related p-ERK1/2; however, both the CatSper and KSper currents, which are thought to interactively regulate Ca^{2+} influx in sperm, were not affected by matrine. **Conclusion:** Our findings indicate that matrine inhibits mouse sperm function by reducing sperm $[Ca^{2+}]_i$ and suppressing the phosphorylation of ERK1/2.

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Introduction

Matrine is a tetracyclo-quinolizidine alkaloid primarily isolated from the roots of *Sophora flavescens* (*kushen*) and *Sophora subprostrata* (*shandougen*), legumes that are used for traditional Chinese medicine [1]. An intensive investigation into the pharmacology and clinical applications of matrine has been ongoing in China for the past decade [2-6]. Matrine has been widely used for the treatment of chronic hepatitis B and chronic hepatitis C since 1998 and has recently been reported to have anti-tumor activities with potential as an adjunct therapy for cancers [5, 7-11]. Based on its effects on potassium and calcium currents as well as intracellular calcium concentration ($[Ca^{2+}]_i$) in rat ventricular myocytes and mouse atrial cardiomyocytes, matrine has also been used for the treatment of heart arrhythmias [12-14]. Clearly, matrine is a research focus in Chinese medicine.

Adverse effect examinations of matrine have mainly focused on the hemic, digestive and renal systems. After matrine injection, blood analyses and renal function tests did not show abnormalities, and adverse reactions were limited to the injection sites [15-17]. Although several Chinese herbs that possess similar bioactivities and a similar biochemical structure to matrine have been shown to have spermicidal activity *in vitro* [18], the effect of matrine on the male reproductive system has not been investigated. Two trends make the examination of matrine's reproductive toxicity necessary and valuable. First, matrine had recently become the main ingredient of medicines for gynecologic inflammation treatment and feminine cleansing in China [19]. Secondly, matrine is also a major component of some biopesticides sprayed in large areas of China [20]. Therefore, determining the effects of matrine on the reproductive system is essential not only for further expanding the knowledge of the side effects of matrine in clinical applications, but also for evaluating the environmental safety of this alkaloid in botanical pesticides.

In this study, we focused on assessing the *in vitro* toxicity of matrine on the viability, motility, capacitation and acrosome reaction of mouse sperm. To understand the mechanisms related to matrine's effects on the reproductive system, its effects on sperm intracellular calcium concentration, sperm-specific ion channels and extracellular signal regulated kinases 1/2 (ERK1/2) were also examined.

Materials and Methods

Animals and treatments

The C57/BL6 mice were purchased from the Animal Center of Jiangxi University of Traditional Chinese Medicine in Nanchang, China. Approval from the Ethics and Animal Care and Use Committee from the same institution was obtained. The male C57/BL6 mice (25-35 g; 12-14 weeks) were housed at a temperature of 20~25°C and under a 12/12 h light/dark schedule. In this study, matrine was administrated by *in vitro* exposure: sperm were released from the cauda epididymis of the mice, as described previously [21], and incubated in human tubal fluid (HTF) medium (Millipore, USA) containing 0, 10, 50, 100 and 200 µM matrine in a 37 °C, 5% CO₂ incubator. Matrine (purity >95%) was purchased from the National Institutes for Food and Drug Control (China).

Assessment of sperm viability

Mouse cauda epididymis sperm were incubated in HTF medium containing different concentrations of matrine in a 37 °C, 5% CO₂ incubator for 2 h. Sperm viability was examined according to ref. [22]. Briefly, 5 µl of each sperm sample was mixed with 5 µl of eosin-nigrosin staining solution at room temperature for 30 s. The mixture was transferred to a slide and imaged using a Leica DM2500 Upright Microscope under the bright field. The head of the dead sperm were stained red, while viable sperm were not stained. For each sample, a minimum of 200 sperm were counted.

Assessment of sperm motility

After incubating the sperm in HTF medium containing different concentrations of matrine for 1 h, aliquots of 5 µl of sperm from each sample were analyzed using a Computer-Assisted Sperm Analysis (CASA)

machine (WLJY-9000, WeiLi. Co., Ltd., China). Three parameters (total sperm motility, progressive motility and linear velocity) were recorded. A minimum of 200 sperm were counted for each assay.

Evaluation of acrosome reaction and capacitation

For the Ca^{2+} ionophore A23187 induced-acrosome reaction, sperm were first capacitated in HTF medium containing different concentrations of matrine for 60 min; then, $10\ \mu\text{M}$ A23187 was added, and the solution was incubated for an additional 30 min. Acrosome reaction and capacitation were assessed using chlortetracycline (CTC) staining as previously described [23]: 'F' is classified as uniform yellow fluorescence in the head, which is characteristic of uncapacitated cells; 'B' is classified by a fluorescence-free band in the post-acrosomal region, which is characteristic of capacitated but acrosome-intact cells; 'AR' is classified as very weak or absent fluorescence, which is characteristic of acrosome-reacted cells. Bright fluorescence in the mid-piece was seen for all cells [23]. Typical examples of these images are shown in Fig. 2A. The stained sperm were examined using a Leica DM2500 Upright Microscope with an Hg excitation beam filtered with a 340-380 nm filter and fluorescence emission collected by a DM 400 dichromatic mirror (Leica "A" filter, Germany). A total of 200 spermatozoa were counted to assess CTC staining patterns. Capacitated sperm were calculated as the sum of "F" and "B".

In vitro fertilization

Female mice were superovulated with 10 IU of pregnant mare serum gonadotropin (PMSG) at 48 hours before egg collection. After 13~15 h post-human chorionic gonadotropin (hCG, 10 IU) administration, oocytes were collected from the cumulus-oocyte complex (COC) and carefully moved into two groups of fertilization drops made by the G-IVF™ PLUS medium (Vitrolife, Sweden) containing 0 (control) and $200\ \mu\text{M}$ matrine. Then, approximate 1×10^5 capacitated sperm that had been incubated in HTF media for 1 h were transferred to the fertilization drops and incubated for 4 h at 37°C . After fertilization for 4 h, the fertilized oocytes were washed with G-IVF™ PLUS medium and cultured for 20 h. The percentage of two-cell embryos was considered the IVF rate.

Single sperm $[\text{Ca}^{2+}]_i$ imaging

Single sperm $[\text{Ca}^{2+}]_i$ imaging was performed as previously described [24]. Briefly, sperm were maintained in HS solution (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) after swimming out of the cauda epididymal and were stained with $5\ \mu\text{M}$ Fluo-4 AM (Molecular Probes, USA) and 0.05% pluronic F-127 (Molecular Probes, USA) for 30 min at room temperature in the dark, followed by washing in HS medium or no Ca^{2+} HS medium containing 2 mM EGTA and no Ca^{2+} . The washed sperm were loaded on Cell-Tak (BD™ Biosciences, USA) coated coverslips of glass bottom cell culture dishes (Φ , 1.5 cm, Nest Biotechnology Co., Ltd.) and were allowed to attach for 20 min. To image the sperm, a monochromator (Polychrome V, TILL Photonics GmbH) was used to generate an excitation at 488 nm for Fluo-4. A $40\times$ objective on an inverted microscope (IX-71, Olympus) was used for imaging. Emissions (515-565 nm) were bandpass filtered (HQ540/50, Chroma) and collected with a cooled CCD camera (CoolSNAP HQ, Roper Scientific) that recorded 100 ms every 2 s. The images were analyzed using commercial software (MetaFluor v7, Molecular Devices).

Western blot

For examining the levels of p-ERK1/2 in mouse sperm, cauda epididymal sperm were incubated with different concentrations of matrine in HTF for 30 min. Sperm proteins were isolated from cauda epididymal sperm according to ref. [21]. A phosphatase inhibitor was added to the lysis buffer to eliminate phosphatase activity. Protein concentration was determined using the BCA method (Thermo Scientific, USA), and 50 μg protein samples were electrophoresed on 10 % SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (GE Healthcare, USA). After incubation with p-Erk1/2 (Thr202/Tyr204) antibodies (Cell Signaling Technology, USA) and HRP-conjugated goat anti-rabbit IgG (Thermo Scientific, USA), the filter was visualized using the ECL detection kit (Thermo Scientific, USA).

Sperm patch-clamping recordings

Whole-cell currents were recorded using the sperm cytosolic droplet method described previously [25, 26]. HS solution was used for sperm swim-out from the corpus epididymis. For KSper recording, the

Fig. 1. The effect of matrine on sperm motility and viability *in vitro*. Cauda epididymal sperm of mouse were incubated with different doses of matrine in HTF medium at 37 °C and 5% CO₂ incubator. After 60 min, the motion parameters (A-C) were analyzed by Computer-Assisted Sperm Analysis (CASA). For assessing the sperm viability (D), the incubation time is extended to 120 min and incubated sperm were stained with eosin-nigrosin solution. A minimum of 200 sperm were counted for each assay. Bar: mean±SD. *P<0.05, ***p<0.0001, *t*-test, *n*=8 to 9.

different doses of matrine were prepared in an extracellular control solution containing 160 mM KOH, 10 mM Hepes, 150 mM methanesulfonic acid (Mes) and 2 mM Ca(Mes)₂, adjusted to pH 7.4 with Mes. The symmetrical 160 mM K⁺ pipette solution contained 155 mM KOH, 5 mM KCl, 10 mM 1,2-bis(2-aminophenoxy)ethane-N, N, N', N'-tetraacetic acid (BAPTA), 20 mM Hepes, and 115 mM Mes with pH 8.0 adjusted with KOH. For recording the monovalent current of CatSper, the matrine stock was diluted with a sodium-based divalent-free (DVF) solution (150 mM NaCl, 20 mM Hepes, and 5 EDTA, pH 7.4) for perfusion. The pipette solution contained 135 mM Cs-Mes, 10 mM Hepes, 10 mM EGTA, and 5 mM CsCl adjusted to pH 7.2 with CsOH. Solutions were applied directly via a local perfusion system for 45-60 s. The currents were analyzed with Clampfit (Axon, Gilze, Netherlands) and Grapher 8 softwares (Golden Software, Inc., Golden, Co, USA).

Statistical analysis

Data are expressed as means±SD. Differences between the control and samples were assessed by an unpaired *t* test. Differences with a P<0.05 were considered statistically significant.

Results

Matrine inhibits mouse sperm mobility without affecting mouse sperm viability

In this study, we assessed the effect of matrine on mouse sperm motility, which is an important function for fertilization [27]. After incubation with different concentrations of matrine in HTF medium in a 37 °C, 5% CO₂ incubator for 1 h, sperm motility was determined using computer-assisted sperm analysis (CASA). The three motility parameters, including total sperm motility, progressive motility and linear velocity, were significantly lower in the samples treated with 100 and 200 μM matrine *in vitro* than in the control (Fig. 1A-C). As previously reported, several Chinese herbs that possess bioactivities similar to matrine have spermicidal activity *in vitro* [18]. Thus, we examined whether matrine affected the viability of mouse sperm after *in vitro* incubation. The results showed that mouse sperm viability

Fig. 2. Assessment of the acrosomal status of mouse sperm effect incubated with different dose of matrine *in vitro*. A, the different CTC staining patterns: “F”, characteristic of uncapacitated sperm; “B”, representing capacitated but acrosome-intact sperm; and “AR”, corresponding to sperm that have undergone acrosomal exocytosis. B, the effect of matrine on Ca^{2+} ionophore A23187 induced-acrosome reaction of mouse sperm. Sperm were first capacitated in HTF medium containing different concentration of matrine for 60 min and then 10 μM A23187 was added for another 30 min incubation. Bar: mean $\pm$ SD. *** $p < 0.0001$, t -test, $n = 9$. C, the effect of matrine on spontaneous acrosome reaction of mouse sperm. Sperm were capacitated in HTF medium containing different concentration of matrine for 60 min and directly stained by CTC. $n = 9$. D, the effect of matrine on mouse sperm capacitation. Sperm were capacitated in HTF medium containing different concentration of matrine for 60 min and directly stained by CTC. Capacitated sperm were calculated as the sum of “F” and “B”. $n = 9$.

is not significantly affected by *in vitro* exposure to matrine (Fig. 1D), suggesting that the compromised sperm motility was not a result of a decrease in sperm viability.

Matrine lowers A23187-induced acrosome reaction of mouse sperm

The acrosome reaction (AR) is a Ca^{2+} -dependent exocytosis process essential for sperm penetrating the oocyte [28]. *In vitro*, it can be induced through co-incubation of sperm with the calcium ionophore A23187. In addition, a certain percentage of sperm will undergo acrosome reaction spontaneously. Thus, in this study, we assessed the effects of matrine on both spontaneous and A23187-induced AR. Furthermore, we also examined if matrine affected *in vitro* capacitation, which is also a Ca^{2+} -dependent process. A chlortetracycline (CTC) staining assay was used to characterize both capacitation and the acrosome reaction. The CTC staining patterns (“F”, “B” and “AR”) correspond to different acrosomal statuses and are shown in Fig. 2A. The results showed that A23187-induced AR of mice sperm was remarkably decreased by co-incubation with matrine *in vitro* at the doses of 100 and 200 μM (Fig. 2B), while spontaneous AR (Fig. 2C) and capacitation (Fig. 2D) of mice sperm were not altered at any matrine doses.

Matrine decreases the *in vitro* fertilization rate of mouse sperm

In vitro fertilization can be used to evaluate the comprehensive ability of sperm to fertilize oocytes. Thus, we examined the fertilization rate of mouse sperm exposed to

Fig. 3. The effects of matrine on the *in vitro* fertilization rate of mouse sperm. Cauda epididymis sperm of mice were capacitated in HTF medium for 60 min and then transferred and incubated with eggs collected from superovulated female mice in the fertilization drops made by the G-IVF™ PLUS medium (Vitrolife, Sweden) with (+Matrine) or without 200 μ M matrine (-Matrine), respectively. The 2-cell embryos in the absence of sperm (-Sperm) were used as the negative controls. The percentage of two-cell embryos (A) was calculated as the IVF rate. B, The statistical analysis of percentage (B). Bar: mean $\pm$ SD. * $p < 0.05$, *t*-test, $n = 4$. ND, not detected.

200 μ M matrine, a dose that can significantly inhibit mouse sperm function, *in-vitro* (Fig. 1 and 2). Oocytes harvested from super-ovulating females were incubated with 200 μ M matrine treated sperm, and the fertilization rate was significantly lower than for the control (Fig. 3). The results from four trials with total 111 oocytes showed that averagely 64.83 % of oocytes were successfully fertilized by matrine-untreated sperm. Sperm exposure to 200 μ M matrine resulted in a significant reduction of fertilization ratio 36.88 % with 98 oocytes examined. Considering the possible parthenogenetic effect, as a negative control, we detected the 2-cell embryos in the absence of sperm. Interestingly, although usually there is about 5% parthenogenetic embryos in the IVF tests [29], in our conditions there was no 2-cell embryos observed in the absence of sperm, either with or without matrine (68 and 75 oocytes examined, respectively).

Matrine reduces the intracellular Ca^{2+} concentration of mouse sperm

The present study demonstrated that the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)-dependent sperm functions, such as motility (Fig. 1) and the acrosome reaction (Fig. 2B), were inhibited by matrine, implying that matrine may affect sperm Ca^{2+} signaling. In fact, there were reports showing that matrine could alter $[Ca^{2+}]_i$ in ventricular myocytes [12, 13]. Therefore, we used a single sperm $[Ca^{2+}]_i$ imaging assay to determine whether matrine affects mouse sperm $[Ca^{2+}]_i$. First, we examined the effects of matrine on sperm $[Ca^{2+}]_i$ in HS medium containing 2 mM Ca^{2+} . The results indicated that matrine at doses of 100 and 200 μ M, which inhibit mouse sperm function (Fig. 1-3), also cause a reduction of sperm $[Ca^{2+}]_i$ within 1 min compared with control (Fig. 4A and B). This reduction presented significantly in the head (Fig. 4A). However, the sperm $[Ca^{2+}]_i$ was not statistically different between sperm perfused with 10 and 50 μ M matrine and the control (Fig. 4A-C). These results are consistent with our sperm function analysis (Fig. 1-3). It is worth to point out that, in the end of each experiment 10 μ M of the calcium ionophore A23187 was added to confirm the vitality of the sperm. Because a drop of $[Ca^{2+}]_i$ could be resulted from either an inhibition of Ca^{2+} -increasing pathways (such as Ca^{2+} entry through Ca^{2+} channels) or a stimulation of Ca^{2+} -decreasing

Fig. 4. The effects of different doses of matrine on $[Ca^{2+}]_i$ of mouse sperm. Mouse sperm were stained with 5 μM Fluo4-AM. The changes of sperm $[Ca^{2+}]_i$ were calculated as $\Delta F/F_0$ (F_0 , the mean fluorescence intensity of the sperm head before adding matrine; F , the fluorescence of the sperm head after adding matrine; $\Delta F = F - F_0$). A, Examples of single cell $[Ca^{2+}]_i$ were illustrated below the graphs with the time frame indicated in Fig. 4B. B, The time-course changes of sperm $[Ca^{2+}]_i$ exposed to different doses of matrine in HS medium containing 2 mM Ca^{2+} . C, The statistical analysis of Fig. 4B at the time frame of 100 s. D, The statistical analysis of sperm $[Ca^{2+}]_i$ exposed to different doses of matrine in Ca-depleted HS medium. $n = 3$ batches for B-D, 10 to 12 sperms for each batch. Bar: mean $\pm$ SD. *** $p < 0.0001$, t-test.

pathways (such as Ca^{2+} exclusion by plasma membrane Ca^{2+} pumps), we further investigated the effect of matrine on $[Ca^{2+}]_i$ in a nominally Ca^{2+} free extracellular medium (no Ca^{2+} and 2 mM EGTA added) as a first effort to clarify these two possibilities. The results showed that matrine did not change sperm $[Ca^{2+}]_i$ when there was no extracellular Ca^{2+} (Fig. 4D), indicating that the effect of matrine on $[Ca^{2+}]_i$ is extracellular Ca^{2+} -dependent and favoring the hypothesis of inhibition on Ca^{2+} entry rather than a stimulation on calcium pumps. Overall, these results imply that the inhibitory effect of matrine on mice sperm functions may result from the reduction of mouse sperm $[Ca^{2+}]_i$.

Matrine does not affect ICatSper or IKsper of mouse sperm

Inhibition on Ca^{2+} channels is a general mechanism to reduce $[Ca^{2+}]_i$. As previously reported, matrine could regulate Ca^{2+} currents in ventricular myocytes [12, 13]. Our own results also suggested that matrine may inhibit Ca^{2+} entry (Fig 4). In mouse sperm, CatSper is the predominant ion channel responsible for Ca^{2+} influx [25, 30]. Hence, to determine the mechanism underlying sperm $[Ca^{2+}]_i$ reduction caused by matrine, we examined the effects of matrine on CatSper with whole-cell patch clamping. The results turned out that mouse

Fig. 5. Matrine has no effect on mouse sperm I_{CatSper} and I_{KSper} . The effects of different concentration of matrine (Mat) on mouse sperm I_{CatSper} (A) and I_{KSper} (C) were examined using voltage-clamp ramp protocol from -100 to +100 mV described in Materials and Methods. B and D, the mean currents of mouse CatSper and KSper perfused with different concentration of matrine at 100 mV. Bar: mean $\pm$ SD, $n=10$ sperm patches. ** $P<0.005$ by student's t test.

sperm I_{CatSper} was not changed by up to 200 μM matrine (Fig. 5 A and B), suggesting that other pathways underline the sperm $[\text{Ca}^{2+}]_i$ reduction caused by matrine. In mammalian sperm, KSper, the sperm-specific pH-dependent potassium channel, may modulate sperm Ca^{2+} signaling by affecting the driving force for Ca^{2+} influx or the open probability of CatSper [25, 26, 31]. In addition, there was evidence that matrine could block K^+ current in ventricular myocytes [13]. Therefore, we also examined the effect of matrine on mouse KSper, and showed that matrine had no effect on mouse sperm I_{KSper} either (Fig. 5 C and D).

Matrine suppresses the phosphorylation of ERK1/2

In the present study, we found that 100 and 200 μM matrine significantly decreased mouse sperm $[\text{Ca}^{2+}]_i$ (Fig. 4). As a key second messenger, $[\text{Ca}^{2+}]_i$ modulates a range of activities and signals that are pivotal to sperm functions [27, 32]. Thus, we further examined the phosphorylation of the extracellular signal-regulated kinases (ERK) 1/2, which are the Ca^{2+} -dependent signaling molecules that regulate sperm motility and the acrosome reaction [33]. Western blot analysis revealed that the levels of phosphorylated-ERK1 (Thr202, Fig. 6A and B) and phosphorylated-ERK2 (Tyr204, Fig. 6A and C) were obviously reduced in sperm incubated with 100 and 200 μM matrine relative to the control. To evaluate the maximum ERK1/2 activity in sperm, 100 ng/ml phorbol-12-myristate-13-acetate (PMA) was used to stimulate ERK1/2 to their maximum phosphorylation levels (Fig. 6). On the other hand, 0.5 μM PD0325901 was applied to measure the minimum phosphorylation levels of pERK1/2 in this experiments. Roughly, the normal ERK1/2 phosphorylation levels in sperm were about

Fig. 6. The alteration of phospho-ERK1/2 (p-ERK1/2) in mouse sperm exposed to different doses of matrine *in vitro*. Sperm proteins were isolated from cauda epididymal sperm and incubated in HTF medium containing different doses of matrine, or 100 ng/ml Phorbol-12-myristate-13-acetate (PMA) as positive control, or 0.5 μ M PD0325901 as negative control. The phosphorylation of ERK1/2 was measured by western blot using phospho-Erk1/2 (Thr202/Tyr204) antibody (A) and quantified with the intensities of the bands normalized to β -Actin using Image J2x software (B and C). Bar: mean $\pm$ SD, * p < 0.05, ** p < 0.005 by student's *t*-test, n = 3.

half of their maximums, and 200 μ M matrine could reduce them to 25% of their maximum levels (Fig. 6).

Discussion

Matrine has a variety of bioactivities and is an alternative natural product used for clinical and agricultural applications in China. Limited reports revealed that this alkaloid has few side effect. For example, blood analyses and renal function tests did not find any toxicity of matrine. However, little attention has been paid to the effect of matrine on the reproductive system. Here, we present the first report of the effects of matrine on mouse sperm *in vitro*. Unlike other structurally similar spermicidal herbs, high concentrations of matrine inhibited mouse sperm functions, including motility (Fig. 1), the acrosome reaction (Fig. 2) and *in vitro* fertilization (Fig. 3), but did not affect mouse sperm viability mouse *in vitro* (Fig. 1). These results imply that severe attention is required when utilizing high doses of matrine in clinical applications.

Mature sperm are fully differentiated cells with highly condensed nuclei that lack active transcriptional machinery [33, 34]. Hence, intracellular signal transduction pathways can serve as important modulators of mammalian sperm functions [33]. Intracellular Ca^{2+} , a key second messenger, plays an essential role in modulating a range of activities that are pivotal to sperm functions [27, 32]. In this study, we found that both 100 and 200 μ M matrine reduced mouse sperm $[\text{Ca}^{2+}]_i$ in the presence of 2 mM extracellular Ca^{2+} , while sperm $[\text{Ca}^{2+}]_i$ was not affected when there was no extracellular Ca^{2+} (Fig. 4), indicating that the inhibition effect of matrine on sperm $[\text{Ca}^{2+}]_i$ is extracellular Ca^{2+} dependent. Interestingly and somewhat surprisingly, our results excluded the possibility that matrine endows effect

through directly blocking CatSper or KSper (Fig. 5), two main ion channels thought to be essential for modulating sperm $[Ca^{2+}]_i$ [25, 26, 30]. In fact, although so far CatSper is the only calcium channel detectable in mature mammalian sperm with patch clamping techniques, the existence of other types of calcium channels in mature mammalian sperm has not been ruled out [27, 35]. On the other hand, besides being the Ca^{2+} resource for Ca^{2+} channels, extracellular Ca^{2+} may be involved in Ca^{2+} -signaling through distinct ways, such as acting as a ligand for certain GPCRs or even regulating calcium pumps [36]. Definitely, further experiments are required to clarify the extracellular Ca^{2+} -dependent mechanisms by which matrine inhibits sperm $[Ca^{2+}]_i$.

ERK1 and ERK2 are related protein-serine/threonine kinases that participate in the regulation of a large variety of processes [37]. In mammalian sperm, these proteins act as regulators of sperm motility and the acrosome reaction [33]. Several previous reports have shown that matrine can inactivate ERK1/2 in various cancer cells *in vitro* [9, 11, 38]. In this study, we found that matrine also inactivates ERK1/2 signaling by suppressing the phosphorylation of ERK1/2 in mouse sperm (Fig. 6). These results imply that the inhibitory effect of matrine on sperm motility and the acrosome reaction is probably the result of the suppression of the phosphorylation of ERK1/2. In many mammalian cells, the phosphorylation of ERK1/2 is tightly coupled to $[Ca^{2+}]_i$ status. The changes in $[Ca^{2+}]_i$ mediate the phosphorylation of ERK1/2 [27, 39]. In this study, we found that the phosphorylation of ERK1/2 was also associated with $[Ca^{2+}]_i$ in mouse sperm. These results imply that matrine inhibits functions of mouse sperm by reducing sperm $[Ca^{2+}]_i$ and suppressing the phosphorylation of ERK1/2.

In conclusion, our study examined the effect of matrine on mouse sperm function *in vitro* and further elucidated its inhibitory mechanism. Our results indicated consistently that *in-vitro* exposure to matrine inhibits the functions of mouse sperm by reducing sperm $[Ca^{2+}]_i$ and the suppression of phosphorylation of ERK1/2. Our study represents a significant mechanistic advancement in the understanding of the reproductive toxicity of matrine. This will help determine the appropriate spectrum of practical applications for matrine.

Disclosure Statement

The authors have no conflicts of interest to declare.

Acknowledgments

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References

- 1 Niu K: Pharmacology and clinical application of sophora flavescens. *Int J Oriental Med* 1997;22:75-81.
- 2 Zhang JP, Zhang M, Zhou JP, Liu FT, Zhou B, Xie WF, Guo C: Antifibrotic effects of matrine on in vitro and in vivo models of liver fibrosis in rats. *Acta Pharmacol Sinica* 2001;22:183-186.

- 3 Long Y, Lin XT, Zeng KL, Zhang L: Efficacy of intramuscular matrine in the treatment of chronic hepatitis B. *Hepatobiliary Pancreat Dis Int* 2004;3:69-72.
- 4 Li X, Chu W, Liu J, Xue X, Lu Y, Shan H, Yang B: Antiarrhythmic properties of long-term treatment with matrine in arrhythmic rat induced by coronary ligation. *Biol Pharm Bull* 2009;32:1521-1526.
- 5 Liu Y, Xu Y, Ji W, Li X, Sun B, Gao Q, Su C: Anti-tumor activities of matrine and oxymatrine: Literature review. *Tumour Biol* 2014;35:5111-119.
- 6 Basile A, Pascale M, Franceschelli S, Nieddu E, Mazzei MT, Fossa P, Turco MC, Mazzei M: Matrine modulates hsc70 levels and rescues $\Delta F508$ -CFTR. *J Cell Physiol* 2012;227:3317-3323.
- 7 Zhang S, Zhang Y, Zhuang Y, Wang J, Ye J, Zhang S, Wu J, Yu K, Han Y: Matrine induces apoptosis in human acute myeloid leukemia cells via the mitochondrial pathway and akt inactivation. *PloS one* 2012;7:e46853.
- 8 Luo C, Zhu Y, Jiang T, Lu X, Zhang W, Jing Q, Li J, Pang L, Chen K, Qiu F, Yu X, Yang J, Huang J: Matrine induced gastric cancer mkn45 cells apoptosis via increasing pro-apoptotic molecules of bcl-2 family. *Toxicology* 2007;229:245-252.
- 9 Liu X, Jiang J, Jiao X, Wu Y, Lin J: Matrine-induced apoptosis in leukemia u937 cells: Involvement of caspases activation and mapk-independent pathways. *Planta Med* 2006;72:501-506.
- 10 Li LQ, Li XL, Wang L, Du WJ, Guo R, Liang HH, Liu X, Liang DS, Lu YJ, Shan HL, Jiang HC: Matrine inhibits breast cancer growth via mir-21/pten/akt pathway in mcf-7 cells. *Cell Physiol Biochem* 2012;30:631-641.
- 11 Xie M, Yi X, Wang R, Wang L, He G, Zhu M, Qi C, Liu Y, Ye Y, Tan S, Tang A: 14-thienyl methylene matrine (yyj18), the derivative from matrine, induces apoptosis of human nasopharyngeal carcinoma cells by targeting mapk and pi3k/akt pathways in vitro. *Cell Physiol Biochem* 2014;33:1475-1483.
- 12 Shan H, Yang B, Zhou Y, Wang H, Li B: Effects of matrine on aconitine-induced electrophysiological changes in rat ventricular myocytes. *J Chin Pharmaceu Sci* 2004;13:193-198.
- 13 Zhou Y, Xu W, Han R, Zhou J, Pan Z, Rong H, Li J, Xu C, Qiao G, Lu Y: Matrine inhibits pacing induced atrial fibrillation by modulating $i(\text{km}3)$ and $i(\text{ca-l})$. *Int J Biol Sci* 2012;8:150-158.
- 14 Hu MQ, Dong ZX, Zhao WX, Sun J, Zhao X, Gu DF, Zhang Y, Li BX, Yang BF: The novel mechanism of oxymatrine affecting herg currents at different temperatures. *Cell Physiol Biochem* 2010;26:513-522.
- 15 Zhang L, Liu W, Zhang R, Wang Z, Shen Z, Chen X, Bi K: Pharmacokinetic study of matrine, oxymatrine and oxysophocarpine in rat plasma after oral administration of sophora flavescens ait. Extract by liquid chromatography tandem mass spectrometry. *J Pharmaceut Biomed* 2008;47:892-898.
- 16 Espino J, Mediero M, Lozano GM, Bejarano I, Ortiz A, Garcia JF, Pariente JA, Rodriguez AB: Reduced levels of intracellular calcium releasing in spermatozoa from asthenozoospermic patients. *Reprod Biol Endocrinol* 2009;7:11.
- 17 Zhang XL, Xu HR, Chen WL, Chu NN, Li XN, Liu GY, Yu C: Matrine determination and pharmacokinetics in human plasma using lc/ms/ms. *J Chromatogr B* 2009;877:3253-3256.
- 18 Ogbuewu IP, Unamba-Oparah IC, Odoemenam VU, Etuk IF, Okoli IC: The potentiality of medicinal plants as the source of new contraceptive principles in males. *N Am J Med Sci* 2011;3:255-263.
- 19 Cao HW, Zhang H, Chen ZB, Wu ZJ, Cui YD: Chinese traditional medicine matrine: A review of its antitumor activities. *J Med Plants Res* 2011;5:1806-1811.
- 20 Lim SJ, Jeong DY, Choi GH, Park BJ, Kim JH: Quantitative analysis of matrine and oxymatrine in sophora flavescens extract and its biopesticides by uplc. *J Agr Chem Environ* 2014;3:64-73.
- 21 Yang C, Zeng X-H, Zhou Y, Xia X-M, Lingle CJ: Lrrc52 (leucine-rich-repeat-containing protein 52), a testis-specific auxiliary subunit of the alkalization-activated slo3 channel. *Proc Natl Acad Sci U S A* 2011;108:19419-19424.
- 22 Bjorndahl L, Soderlund I, Kvist U: Evaluation of the one-step eosin-nigrosin staining technique for human sperm vitality assessment. *Hum Reprod* 2003;18:813-816.
- 23 Shi QX, Roldan ER: Bicarbonate/co2 is not required for zona pellucida- or progesterone-induced acrosomal exocytosis of mouse spermatozoa but is essential for capacitation. *Biol Reprod* 1995;52:540-546.
- 24 Xia J, Ren D: Egg coat proteins activate calcium entry into mouse sperm via catsper channels. *Biol Reprod* 2009;80:1092-1098.
- 25 Kirichok Y, Navarro B, Clapham DE: Whole-cell patch-clamp measurements of spermatozoa reveal an alkaline-activated ca^{2+} channel. *Nature* 2006;439:737-740.
- 26 Zeng XH, Yang C, Kim ST, Lingle CJ, Xia XM: Deletion of the slo3 gene abolishes alkalization-activated k^{+} current in mouse spermatozoa. *Proc Natl Acad Sci U S A* 2011;108:5879-5884.

- 27 Publicover SJ, Giojalas LC, Teves ME, de Oliveira G, Garcia AAM, Barratt CLR, Harper CV: Ca²⁺ signalling in the control of motility and guidance in mammalian sperm. *Front Biosci* 2008;13:5623-5637.
- 28 Yanagimachi R: Mammalian sperm acrosome reaction: Where does it begin before fertilization? *Biol Reprod* 2011;85:4-5.
- 29 Surani MA, Barton SC: Development of gynogenetic eggs in the mouse: Implications for parthenogenetic embryos. *Science* 1983;222:1034-1036.
- 30 Carlson AE, Westenbroek RE, Quill T, Ren D, Clapham DE, Hille B, Garbers DL, Babcock DF: Catsper1 required for evoked Ca²⁺ entry and control of flagellar function in sperm. *Proc Natl Acad Sci U S A* 2003;100:14864-14868.
- 31 Navarro B, Kirichok Y, Clapham DE: K_{sper}, a pH-sensitive K⁺ current that controls sperm membrane potential. *Proc Natl Acad Sci U S A* 2007;104:7688-7692.
- 32 Publicover S, Harper CV, Barratt C: [Ca²⁺]_i signalling in sperm - making the most of what you've got. *Nat Cell Biol* 2007;9:235-242.
- 33 Almog T, Lazar S, Reiss N, Etkovitz N, Milch E, Rahamim N, Dobkin-Bekman M, Rotem R, Kalina M, Ramon J, Raziel A, Brietbart H, Seger R, Naor Z: Identification of extracellular signal-regulated kinase 1/2 and p38 mapk as regulators of human sperm motility and acrosome reaction and as predictors of poor spermatozoan quality. *J Biol Chem* 2008;283:14479-14489.
- 34 Abou-haila A, Tulsiani DRP: Signal transduction pathways that regulate sperm capacitation and the acrosome reaction. *Arch Biochem Biophys* 2009;485:72-81.
- 35 Lishko PV, Kirichok Y, Ren D, Navarro B, Chung J-J, Clapham DE: The control of male fertility by spermatozoan ion channels. *Annu Rev Physiol* 2012;74:453-475.
- 36 Carafoli E: Calcium signaling: A tale for all seasons. *Proc Natl Acad Sci U S A* 2002;99:1115-1122.
- 37 Roskoski R, Jr.: Erk1/2 map kinases: Structure, function, and regulation. *Pharmacol Res* 2012;66:105-143.
- 38 Jiang T, Zhu Y, Luo C, Lu X, Zhang W, Qiu F, Huang J: Matrine inhibits the activity of translation factor eIF4e through dephosphorylation of 4E-BP1 in gastric MKN45 cells. *Planta Med* 2007;73:1176-1181.
- 39 Sandoval AJ, Riquelme JP, Carretta MD, Hancke JL, Hidalgo MA, Burgos RA: Store-operated calcium entry mediates intracellular alkalization, Erk1/2, and Akt/PKB phosphorylation in bovine neutrophils. *J Leukocyte Biol* 2007;82:1266-1277.