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Rosmarinic acid compromises human sperm functions by an intracellular Ca^{2+} concentration-related mechanism

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ABSTRACT

Rosmarinic acid (RA), a natural phenolic ester, is cytoprotective for male reproduction in animal models. The present study investigated the *in vitro* actions of RA on human sperm functions. Human sperm were exposed to 1, 10, 100, and 1000 μM RA *in vitro* and sperm functions were examined. The results showed that although RA did not affect human sperm viability, RA at 10–1000 μM dose-dependently reduced sperm motility, penetration ability, capacitation, and spontaneous acrosome reaction. In addition, the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), which serve as a key regulator of sperm function, was decreased by RA (10–1000 μM) in a dose-dependent manner. Furthermore, the current of the sperm-specific potassium channel, KSPER, which is predominant for Ca^{2+} influx in sperm, was dose-dependently inhibited by 10–1000 μM RA. Therefore, we conclude that *in vitro* exposure to RA can compromise human sperm functions by decreasing sperm $[Ca^{2+}]_i$ through the suppression of KSPER current.

1. Introduction

Rosmarinic acid (RA) is a phenolic ester derived from caffeic acid and (*R*)-(+)-3-(3,4-dihydroxyphenyl)lactic acid. RA accumulates in high amounts in many plant species, and is especially abundant in many herbs of the Lamiaceae family, such as rosemary, mint, and basil [1,2]. The numerous biological and pharmacological activities of RA that have led to many clinical applications include antioxidant, antitumor, anti-fibrosis, anti-inflammatory, antimicrobial, and anti-neurodegenerative disease activities [1–5]. In addition, RA has long been used in the food, cosmetic, and fragrance industries [6–8]. The pharmacology and biotechnological applications of RA have been intensively studied during the last decade.

Recent studies have reported that RA can attenuate doxorubicin-induced testicular injury and reverse the negative effects of metronidazole-induced male infertility in rats [9,10]. RA also increases sexual behavior and testosterone levels in diabetic male rats [11]. In addition, the strong antioxidant activity of RA improves sperm function and *in vitro* fertilizing ability after sperm cryopreservation in several animals including boar [12], bull [13], and ram [14]. These results indicate that RA acts as a cytoprotective agent for male reproduction in animal models. However, the effect of RA on human sperm remains unclear. We recently described that a Chinese herb that has similar bioactivities to RA inhibits human sperm functions by reducing sperm intracellular calcium ion concentration ($[Ca^{2+}]_i$) and tyrosine phosphorylation [15]. Determining the effect of RA on human sperm is essential to expand the knowledge of the clinical adverse effects of RA and to evaluate the safety of RA as a cryopreservative agents of human sperm.

In this study, we evaluated the effects of RA on the viability, motility, capacitation, and acrosome reaction processes of human sperm, which are essential for fertilization. To understand the underlying mechanism, the effects of RA on sperm $[Ca^{2+}]_i$, currents of the cation channel of sperm (CATSPER) and potassium channel of sperm (KSPER), and intracellular cyclic adenosine monophosphate (cAMP) were examined.

2. Materials and methods

2.1. Semen sample collection and treatments

Semen samples were obtained by masturbation after 3–5 days of

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sexual abstinence. The donors (25–38 years old) had reproductive history during the preceding 2 years and normal sperm quality according to the World Health Organization (WHO) laboratory manual for the examination and processing of human semen (http://www.who.int/reproductivehealth/publications/infertility/9789241547789/en/). The collection of the samples was approved by the Institutional Ethics Committee on human subjects of Jiangxi Maternal and Child Health Hospital. All the donors signed the informed written consent before participation in this study. After purification of sperm by direct swim-up in human tubal fluid (HTF) medium (93.8 mM NaCl, 4.69 mM KCl, 0.2 mM $MgSO_4$, 0.37 mM KH_2PO_4, 2.04 mM $CaCl_2$, 21.4 mM lactic acid, 2.78 mM glucose, 21 mM HEPES, 4 mM $NaHCO_3$, and 0.33 mM Na-pyruvate, pH 7.35 with NaOH), the sperm were incubated with 0, 1, 10, 100, or 1000 μM RA for different times and incubation solutions according to the experimental protocols. The doses of RA were based on the previously reported concentrations used in sperm cryopreservation [12]. RA (purity ≥ 98%) was purchased from Sigma Chemical Co. (R4033; St. Louis, MO, USA).

2.2. Assessment of sperm viability and motility

Sperm suspensions were treated with the different concentrations of RA for 1 h in HTF medium (a non-capacitated buffer) and for 4 h in HTF + + medium (HTF plus 25 mM $NaHCO_3$ and 0.4% HSA; a capacitated medium), which represent non-capacitated and capacitated stages, respectively. Sperm viability was examined using eosin staining according to the WHO laboratory manual for the examination and processing of human semen. The heads of dead sperm were stained red, while viable sperm were not stained. After incubation, an aliquot of 10 μl of each sperm sample was transferred to a sperm-counting chamber (Sefi-Medical Instruments Ltd., Haifa, Israel) and sperm motility parameters were assessed using a Computer-Assisted Sperm Analysis (CASA) system (WLJY-9000; WeiLi Co., Ltd., Beijing, China) as described previously [16]. At least, 200 sperm were counted for each assay.

2.3. Penetration of artificial viscous medium

Human sperm were first capacitated in HTF + + medium for 4 h and then incubated with different doses of RA and 10 μM progesterone (P4, a positive control) for 0.5 h at 37 °C in a 5% CO_2 incubator. The examination of human sperm penetration into viscous media (1% methylcellulose prepared in HTF + + medium) was performed as previously described [17]. Briefly, the open ends of the 7.5-cm flattened capillary tubes with 1.0-mm inner depth (Elite Medical Co., Ltd., Nanjing, China) were inserted into RA- or progesterone-incubated sperm samples for 1 h. Then, the tubes were removed, wiped and observed using a CASA system. Three fields at 1 and 2 cm from the base of the tube were counted. The average number of cells per field was calculated and normalized to values from parallel, untreated controls.

2.4. Evaluation of capacitation and acrosome reaction

Human sperm were exposed to RA and 10 μM A23187 (a positive control) in HTF + + medium for 4 h, respectively. Capacitation and acrosome reaction of human sperm were detected by a 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) staining method as described previously [18]. The stained sperms were examined with a Leica DM2500 Upright Microscope using an Hg excitation beam passed through a 340–380 nm filter and fluorescence emission *via* a DM 400 dichromatic mirror (Leica "A" filter, Germany). A total of 200 sperm were counted to assess the different CTC staining patterns as follows: "F" represents the characteristics of uncapacitated sperm; "B" represents the capacitated but acrosome-intact sperm; and "AR" corresponds to sperms that had undergone acrosomal exocytosis. The capacitation and acrosome reaction were quantified as the percentage of AR- and B-patterned cells and the percentage of AR-patterned cells in the 200 examined sperm, respectively.

2.5. Measurement of sperm $[Ca^{2+}]_i$

The change of human sperm $[Ca^{2+}]_i$ was measured using the fluorescent Ca^{2+} indicator Fluo-4 AM (Molecular Probes, Eugene, OR, USA) by single-sperm $[Ca^{2+}]_i$ imaging as previously described [15]. The change of sperm $[Ca^{2+}]_i$ was calculated by $\Delta F/F_0$ (%) indicating the percent (%) of fluorescent changes (ΔF) normalized to the mean basal fluorescence before the application of RA (F_0). $\Delta F/F_0$ (%) = $(F - F_0)/F_0 \times 100\%$, F indicates the fluorescent intensity at each recorded time point.

2.6. Determination of intracellular cAMP content

The intracellular cAMP content was determined using a competitive immunoassay as described previously [16]. Human sperm were incubated with RA in high-saline (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) at 37 °C in a 5% CO_2 incubator for 1 h. The suspension was quenched with 0.5 M $HClO_4$ and were frozen in liquid N2, thawed, and neutralized by the addition of 0.24 M K_3PO_4. The salt precipitate and cell debris were pelleted by centrifugation at 12,000 *g* for 10 min at 4 °C. The cAMP content in the supernatant was examined using a cAMP-Screen cAMP immunoassay system (Thermo Fisher Scientifc, Waltham, MA, USA) in 96-well plates in an EnSpire® Multimode Plate Reader (Perkin Elmer, Waltham, MA, USA) according to the user's manual.

2.7. Sperm patch-clamp recordings

The whole-cell patch-clamp technique was applied to record human sperm CATSPER and KSPER currents as previously described [19,20]. Seals were formed at the sperm cytoplasmic droplet or the neck region by a 15–30 MΩ pipette. Then, the transition into whole-cell mode was made by the application of short (1 ms) voltage pulses (400–650 mV) combined with light suction. The currents were stimulated by 1 s voltage ramps from −100 to + 100 mV from a holding potential of 0 mV. For recording the monovalent current of CATSPER, divalent-free (DVF) solution (150 mM NaCl, 20 mM HEPES, and 5 mM EDTA, pH 7.4) was used to record basal CATSPER monovalent currents. Then, 1000 μM RA in DVF were perfused to record RA-induced CATSPER current. For KSPER recording, cells were perfused with the high-K^+ HS (160 K^+) solution (160 mM KOH, 10 mM HEPES, 150 mM methanesulfonic acid (MES), and 2 mM $Ca(MES)_2$, adjusted to pH 7.4 with MES) and 1–1000 μM RA in 160 K^+ solution to record the basal and RA-induced KSPER currents. Data were analyzed with Clampfit software (Axon, Gilze, the Netherlands).

2.8. Statistical analysis

Data are expressed as mean ± SEM. Differences between the controls and the treated samples were assessed with two-way ANOVA analysis and the Dunnett's test. Statistically significant differences were determined at $P < 0.05$ by the statistical software GraphPad Prism (version 5.01; GraphPad Software, San Diego, CA, USA).

3. Results

3.1. RA treatment affects human sperm motility

To evaluate the effects of RA on sperm viability and motility at non-capacitated and capacitated stages, we incubated human sperm in HTF medium (a non-capacitated solution) for 1 h and in HTF + + medium (a capacitated solution) for 4 h, respectively. The results showed that