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Emodin inhibits human sperm functions by reducing sperm $[Ca^{2+}]_i$ and tyrosine phosphorylation

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ABSTRACT

Emodin, a bioactive anthraquinone widely used in Chinese traditional medicine, disrupts mouse testicular gene expression *in vivo*. In this study, we investigated the toxicity of emodin to human sperm *in vitro*. Different doses of emodin (25, 50, 100, 200 and 400 μM) were applied to ejaculated human sperm. The results indicated that 100, 200 and 400 μM emodin significantly inhibited the total motility, progressive motility and linear velocity of human sperm. In addition, sperm's ability to penetrate viscous medium together with progesterone induced capacitation and acrosome reaction was also adversely affected by emodin. In contrast, emodin did not affect sperm viability. Furthermore, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and tyrosine phosphorylation, which serve as key regulators of sperm function, were dose-dependently reduced by emodin (50–400 μM). These results suggest that emodin inhibits human sperm functions by reducing sperm $[Ca^{2+}]_i$ and suppressing tyrosine phosphorylation *in vitro*.

1. Introduction

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is a naturally occurring anthraquinone presented in the rhizomes of numerous plants, such as *Rheum palmatum* and *Polygonum cuspidatum*. Emodin is also produced as a secondary metabolite by molds and lichens [1]. Intensive pharmacological investigations have demonstrated that emodin possesses an array of pharmacological properties including antibacterial [2,3], purgative [4], immunosuppressive [5], vasorelaxant [6], anti-inflammatory [3], hepatoprotective [7], antioxidant [8] and anticancer [9–11] effects. Thus, emodin has been widely used in anti-inflammatory and anticancer drugs in Asia [9,12].

Earlier studies have documented that emodin specifically inhibits protein tyrosine kinases which catalyze the phosphorylation of tyrosine residues in many protein substrates and thereby play important roles in the regulation of cellular functions [13]. In addition, emodin also potentially inhibits the casein kinase II (CK2), which is a highly conserved serine/threonine protein kinase [12]. By efficiently suppressing the related signaling cascades, emodin represses proliferation, transformation and metastasis and induces apoptosis in different tumor cells [14,15].

In mature mammalian sperm, protein phosphorylation in tyrosine and serine/threonine residues is absolutely required for maintenance of capacitation, acrosome reaction and motility [16,17]. Recently, a study of the toxicity of emodin in male reproductive organs/tissues indicated that emodin causes testicular toxicity. Male mice that are orally administered emodin for 5 days at an initial dose of 1000 mg kg^{-1} followed by 50 mg/kg/day exhibit hypospermatogenesis, eosinophilic changes and germ cell apoptosis due to the disruption of the expression of testicular genes [18]. These results demonstrate the *in vivo* inhibitory effect of emodin on male reproduction. In addition, it is reported that male mice lacking CK2 are infertile and exhibit phenotypes of globozoospermia, failed germ cell survival and characteristic modifications of the spermatid nucleus during spermatogenesis [19,20]. These results imply that emodin might be also toxic to mature sperm *via* the inhibition of protein phosphorylation. However, the *in vitro* toxicity of emodin to mature human sperm remains unknown. Therefore, this investigation of the effects of emodin on human sperm function will further elucidate the toxicity of emodin in the male reproductive system.

In this study, we focused on assessing the *in vitro* toxicity of emodin in terms of the motility, hyperactivation, capacitation and acrosome reaction of human sperm by the methods of computer-assisted sperm analysis, methylcellulose penetration assay and chlortetracycline staining, respectively. The possible mechanisms were explored by examining the intracellular calcium

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concentrations and protein-tyrosine phosphorylation in the sperm, which play important roles in the regulation of sperm function.

2. Materials and methods

2.1. Sample collection and treatments

Semen samples were collected by masturbation from healthy donors who had been proven fertility and had sperm counts >40 million/ml, total motilities >70% and progressive motilities >50%. The collection of the samples was approved by the Institutional Ethics Committee on human subjects of the Secondary Affiliated Hospital of Nanchang University. The sperms were harvested by direct swim-up in human tubal fluid (HTF) medium (Millipore, USA) or 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) as described previously [21]. Emodin (purity >98%) was purchased from the National Institutes for Food and Drug Control (China). In this study, 50 μl aliquots of sperm samples were mixed with equal volumes of HTF medium containing different concentrations of emodin to final doses of 25, 50, 100, 200 and 400 μM and incubated at 37 °C in a 5% CO_2 incubator for different times according to the experimental protocols.

2.2. Assessment of sperm viability and motility

Sperm viability was examined after incubation of 1 h, 4 h and 12 h with eosin–nigrosin staining kits (Xinran Biological Technology Co. Ltd., Shanghai, China) according to the user manual. The head of the dead sperms was stained red, while viable sperms were not stained. Sperm motility was analyzed with a computer-assisted sperm analysis (CASA) system (WLJY-9000, WeiLi Co., Ltd., Beijing, China). Three parameters (*i.e.*, total sperm motility, progressive motility and linear velocity) were recorded. A minimum of 200 sperms were counted for each assay.

2.3. Penetration of the artificial viscous medium

Sperm penetration assay into a 1% (w/v) methylcellulose solution mimicing the viscous environment in female reproductive tract was performed as described previously [21]. Briefly, the methylcellulose was made up using HTF medium and was introduced into 7.5-cm flattened capillary tubes (1.0-mm inner depth; Elite Medical Co., Ltd., Nanjing, China), with one end sealed with plasticine. Before penetration, human sperms were first incubated with drugs for 3 h at 37 °C in a 5% CO_2 incubator. Next, the open ends of the capillary tubes were inserted into the samples and incubated (37 °C, 5% CO_2) for 1 h. Then, the tubes were removed, wiped, and imaged with a Leica DM2500 Upright Microscope. Three fields at 1 and 2 cm from the base of the tube were counted, and the average cells/field was calculated. The cell densities were normalized to values from parallel, untreated controls.

2.4. Evaluation of capacitation and the acrosome reaction

Chlortetracycline (CTC) staining was performed to evaluate the capacitation and acrosome reaction as previously described [22]. 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 spermatozoa 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 capacitated sperms were quantified as the sum of "F" and "B".

2.5. Single-sperm $[Ca^{2+}]_i$ imaging

Ca^{2+} signaling in human sperm was examined by single-sperm $[Ca^{2+}]_i$ imaging as previously described [23]. Briefly, the sperms were loaded with 5 μM Fluo-4 AM (Molecular Probes, USA) and 0.05% pluronic F-127 (Molecular Probes, USA) for 30 min at room temperature in the dark and subsequently washed in HTF medium. The washed sperms were loaded on a Cell-Tak (BD™ Biosciences, USA)-coated coverslip in a glass-bottomed cell culture dish (Φ, 1.5 cm, Nest Biotechnology Co., Ltd.) and allowed to attach for 20 min. To image the imaging sperm, a Polychrome V chromator (TILL Photonics GmbH, Germany) generated 488-nm excitation light for the Fluo-4. A 40× objective on an inverted microscope (IX-71, Olympus) was used for imaging. The emissions (515–565 nm) were bandpass filtered (HQ540/50, Chroma) and collected with a cooled CCD camera (CoolSNAP HQ, Roper Scientific). The sperms were recorded for 60 s before the addition of emodin and for 240 s after the emodin administration (50 ms exposure time, 2 s time interval). The results were analyzed with commercial software (MetaFluor v7, Molecular Devices, Sunnyvale, CA, USA).

2.6. Indirect immunofluorescence

The phosphotyrosine residues of the proteins in human sperms exposed to different concentrations of emodin for 4 h were detected using an indirect immunofluorescence assay. The sperm samples were fixed for 30 min in 4% paraformaldehyde and then attached to poly-L-lysine-treated coverslips and blocked with 5% BSA for 1 h followed by incubation with mouse IgG or anti-phosphotyrosine monoclonal antibody 4G10 (1:100) overnight. Subsequently, the sperms were stained with DyLight 488 AffiniPure goat anti-mouse IgG (1:200, EarthOx, USA) and 1 μM DAPI. After washing, the coverslips containing the stained sperms were loaded on a clear slide and examined under an FV1000-IX81 confocal laser scanning biological microscope (Olympus, Japan) with an LD laser (405) for the DAPI and an M_Ar laser (488 nm) for the Dylight 488. The fluorescent intensities of sperm (F) were detected by Image J2x software. Semi-quantitative analyses were performed by $\Delta F/F_{ctrl}$. F_{ctrl} is the mean intensity of 0 μM emodin treated sperm. $\Delta F = F - F_0$. At least 100 sperms were analyzed in 9 independent samples.

2.7. Western blotting

The human sperms were exposed to different concentrations of emodin at 37 °C in a 5% CO_2 incubator for 4 h. The sperm proteins were isolated according to previously published methods [24]. A phosphatase inhibitor was added to the lysis buffer to eliminate the phosphatase activity. The protein concentrations were determined by the BCA method (Thermo Scientific, USA). 50 μg of protein was electrophoresed on a 10% SDS-polyacrylamide gel and transferred onto a polyvinylidenedifluoride membrane (GE Healthcare, USA). After incubation with the anti-phosphotyrosine monoclonal antibody 4G10 (Merck Millipore, Germany) at a 1:1000 dilution overnight and the application of HRP-conjugated goat anti-mouse IgG (Thermo Scientific, USA), the filter was visualized using the ECL detection kit (Thermo Scientific, USA).

2.8. Statistical analyses

The data are expressed as the means ± SEM. Differences between the controls and the different samples were assessed with unpaired *t* tests. Statistically significant differences were determined at $P < 0.05$.