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Original Paper

β-Elemene Inhibits Human Sperm Function by Affecting Sperm Vitality and Intracellular Calcium

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Key Words
β-elemene • Acrosome reaction • $[Ca^{2+}]_I$ progesterone • Sperm motility

Abstract
Background/Aims: β-Elemene is a bioactive sesquiterpene compound that exhibits a potent anti-tumor effect and is used in various clinical applications. However, little is known about its effect on the male reproductive system. The objective of this study was to investigate the *in vitro* actions of β-elemene on human sperm function and elucidate the underlying mechanism. ***Methods:*** The cytotoxicity of β-elemene toward MCF-10A, MDA-MD-231, and A549 cells was evaluated with cell proliferation and colony formation assays. Additionally, human sperm were treated with different concentrations (0, 10, 20, 40, 80, 160, and 320 μM) of β-elemene *in vitro*. The characteristics in human sperm essential for fertilization, including vitality, motility, capacitation, acrosome reaction, responsiveness to progesterone, and intracellular calcium concentration ($[Ca^{2+}]_i$) were examined with a computer-assisted sperm analysis system, chlortetracycline staining, and a fluorescent Ca^{2+} indicator. ***Results:*** A comprehensive evaluation of sperm motility, especially hyperactivated motility, revealed that treatments with 40–320 μM β-elemene decreased human sperm vitality, motility (total motility, progressive motility, and curvilinear velocity), and penetrating ability in a dose-dependent manner, but were non-toxic or minimally toxic toward MCF-10A, MDA-MD-231, and A549 cells. Although 10 and 20 μM β-elemene did not affect sperm vitality and motility, these concentrations increased the spontaneous acrosome reaction and inhibited progesterone-induced sperm functions by affecting sperm $[Ca^{2+}]_i$. ***Conclusion:*** These results suggest that β-elemene inhibits human sperm function by affecting sperm vitality and $[Ca^{2+}]_i$. These observations must be considered when using β-elemene to treat cancer patients who may wish to preserve their fertility.

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Introduction

β-Elemene, a sesquiterpene compound in zedoary turmeric oil extracted from *Curcuma zedoaria*, has been reported to have multiple forms of bioactivity, including anti-microbial, anti-fibrosis, and anti-tumor effects [1, 2]. Because of its potent anti-tumor activity and low toxicity, β-elemene has been approved by the China Food and Drug Administration for treatment of tumors, such as cancer of the brain, ovary, prostate, breast, lung, liver, colon, and other tissues [1-7]. Previous studies revealed that β-elemene affects tumors by arresting the cell cycle, inhibiting cell invasion, and inducing apoptosis [3, 6-12]. Hence, intensive investigations into the pharmacology and clinical applications of β-elemene have been ongoing in China for the past decade.

The long-term use of β-elemene for treating various types of cancer in patients is considered to be relatively safe [2, 13, 14]. However, several side effects have been reported. For example, injections may lead to phlebitis, local pain, shock, fever, and asthma [2, 15, 16]. Oral administration of β-elemene has been associated with relatively minor adverse effects, including nausea, emesis, diarrhea, and anorexia [15, 17]. To date, investigations on the adverse effects of β-elemene have mainly focused on the hemic, digestive, and renal systems. Nevertheless, some traditional Chinese herbs with components that are similar to β-elemene in terms of bioactivity and biochemical structure have been observed to affect sperm vitality and function *in vitro* [18, 19]. Therefore, determining the effects of β-elemene on the reproductive system is essential for a more comprehensive understanding of the side effects of β-elemene in clinical applications.

In this study, we evaluated the effects of β-elemene on the characteristics of human sperm essential for fertilization, including vitality, motility, capacitation, acrosome reaction, and responsiveness to progesterone. The intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was examined to explore the underlying mechanism. Our results may represent a new reference for understanding the side effects of β-elemene in clinical applications and may be relevant for developing new guidelines for the use of β-elemene in clinical practice.

Materials and Methods

Cells and cell culture

The three cell types were used in this study, MCF-10A (normal cell control), MDA-MB-231 (breast cancer cells), and A540 (lung cancer cells), were all obtained from ATCC. MCF-10A cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) / F12 Ham medium supplemented with 5% horse serum, 20 ng/mL epidermal growth factor, 0.5 mg/mL hydrocortisone, 100 ng/mL cholera toxin, and 10 μg/mL insulin. Additionally, MDA-MB-231 and A549 cells were maintained in DMEM medium containing 10% FBS.

Cell viability assay

Cells were seeded at a density of 1, 000 (MCF-10A and MDA-MB-231) or 2, 000 (A549) cells/well in 96-well plates. The next day, cell cultures were treated with β-elemene (0, 40, 80, 160, 320, or 640 μM) or docetaxel (20 or 200 nM; dissolved in DMSO). All cell cultures contained 0.1% DMSO. The docetaxel concentrations used in this study are reported to be cytotoxic [20]. Thus, they served as positive controls. Cell viability was determined using the Cell Proliferation Assay Kit (Abcam, Cambridge, MA) according to the manufacturer's instructions. The SpectraMax M2 plate reader (Molecular Devices, LLC., San Jose, CA) was used to measure the optical density of solutions (492 nm).

Colony formation assay

Cells were seeded at a density of 500 (MCF-10A and MDA-MB-231) and 1, 000 (A549) cells/well in 6-well plates. The next day, cells were treated with the same concentrations of β-elemene and docetaxel as in the cell viability assay. All cell cultures contained 0.1% DMSO. Surviving colonies were counted at 6-7 days after seeding.

Sperm sample collection and treatment

Semen samples were collected from healthy donors following masturbation after 3-5 days of sexual abstinence. Fertility and normal sperm quality were confirmed for all donors according to the criteria in the World Health Organization (WHO) laboratory manual for examining and processing human semen (WHO, 2010). Sample collection was approved by the Institutional Ethics Committee on Human Subjects of the Jiangxi Provincial Maternal and Child Health Hospital. Sperm was harvested based on the direct swim-up technique involving 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 $C_3H_3NaO_3$; pH adjusted to 7.35 with NaOH) as described previously [21]. Sperm samples were incubated in HTF medium (0.1% DMSO) containing 0, 10, 20, 40, 80, 160, or 320 μM β-elemene at 37 °C in a 5% CO_2 incubator for various periods of time according to the experimental protocol. The β-elemene (purity > 99%) used in this study was purchased from the National Institutes for Food and Drug Control (China).

Assessment of sperm vitality *and motility*

Sperm vitality was examined using the eosin-nigrosin staining method, and sperm motility was assessed with the WLJY-9000 computer-assisted sperm analysis (CASA) system (WeiLi Co., Ltd., Beijing, China) as described previously [21].

Penetration of an artificial viscous medium

A sperm penetration assay involving a 1% (w/v) methylcellulose solution mimicking the viscous environment in the female reproductive tract was performed as described previously [21].

Evaluation of capacitation and the acrosome reaction

The capacitation and acrosome reaction of human sperm were evaluated with the chlortetracycline (CTC) staining method as described [21].

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

Changes in human sperm $[Ca^{2+}]_i$ were measured using the Fluo-4 AM fluorescent Ca^{2+} indicator (Molecular Probes, Eugene, OR) with the EnSpire® Multimode Plate Reader (Perkin Elmer, Waltham, MA) as described [21].

Examination of sperm responsiveness to progesterone

Sperm responsiveness to progesterone was examined in terms of enhancement of penetrating ability, induction of capacitation and the acrosome reaction, and increase in $[Ca^{2+}]_i$. To evaluate the effects of β-elemene on the progesterone-induced penetrating ability and acrosome reaction, human sperm were first capacitated for 4 h in HTF medium containing 25 mM $NaHCO_3$ and 0.4% human serum albumin (HSA; Vitrolife Corporation, Göteborg, Sweden). Next, 20 mM progesterone and different concentrations of β-elemene were added, after which samples were incubated for 1 h. To evaluate the effect of β-elemene on progesterone-induced $[Ca^{2+}]_i$ increase, human sperm were first incubated with different concentrations of β-elemene for 15 min and then with 1 μM progesterone.

Statistical analysis

Data are expressed as the mean ± standard error of mean (SEM). Differences between the controls and treated samples were assessed using two-way analysis of variance (ANOVA). Significant differences ($P < 0.05$) were determined using the GraphPad Prism program (version 5.01; GraphPad Software, San Diego, CA).

Results

β-Elemene reduces human sperm vitality and motility

We first examined the anti-tumor effects of β-elemene. The three cell lines, MCF-10A (normal cell control), MDA-MB-231 (breast cancer cells), and A540 (lung cancer cells), were treated with different concentrations of β-elemene or docetaxel (positive control). The cytotoxicity of β-elemene toward these cells was evaluated using MTS and colony formation assays. The results showed obvious cytotoxic effects of β-elemene against both cancer cell lines at β-elemene concentrations of up to 160 μM (Fig. 1B, C, E, and F). These β-elemene concentrations were non-toxic or minimally toxic to MCF-10A (Fig. 1A and D). Moreover, a

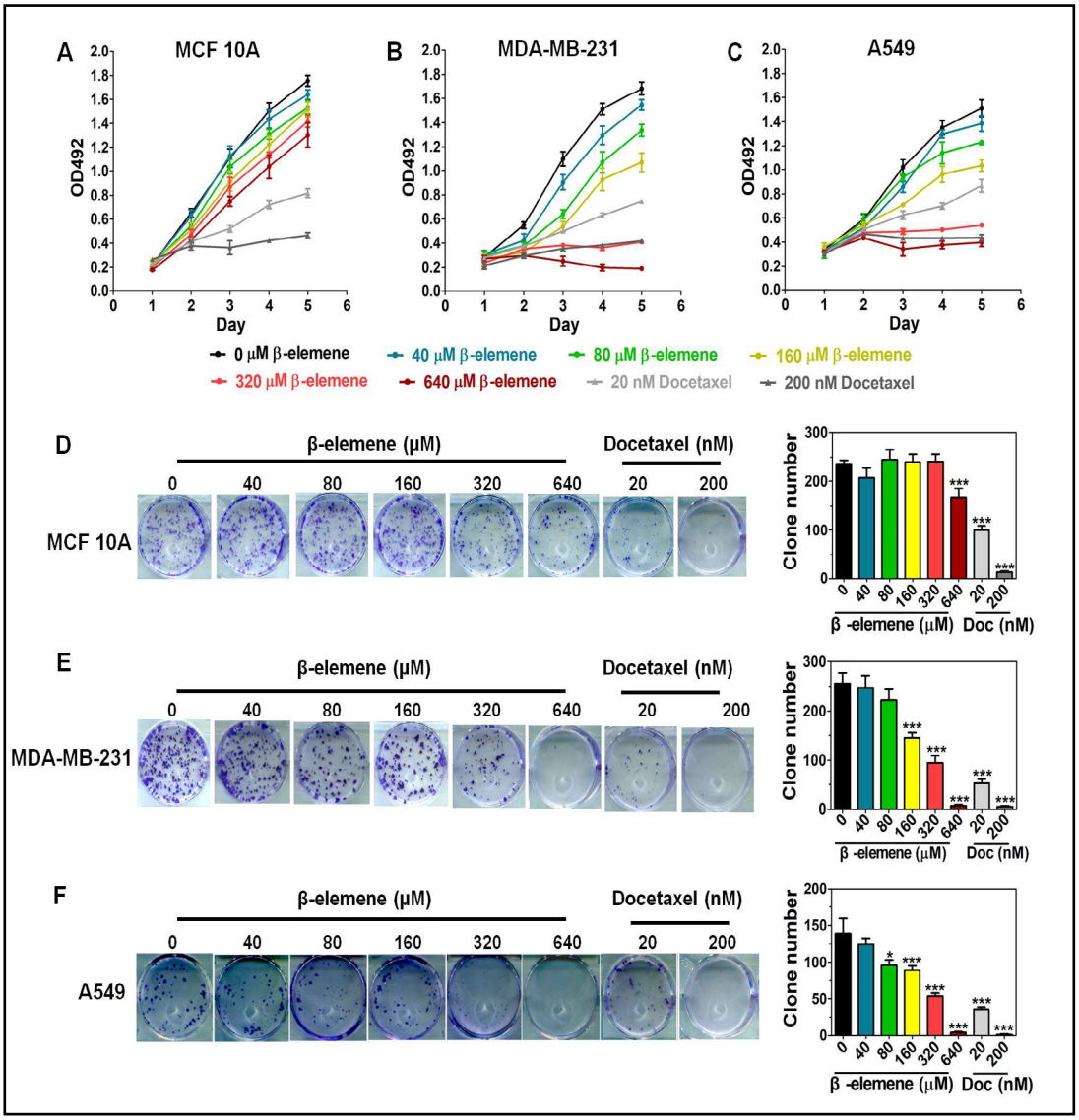

Fig. 1. β-Elemene treatment suppresses viability of cancer cells but not of immortalized normal mammary epithelial cells. (A–C) Growth curves of cells treated with β-elemene or docetaxel at the indicated concentrations. (A) MCF-10A cells (immortalized normal mammary epithelial cells). (B) MDA-MB-231 cells (breast cancer cells). (C) A549 cells (lung cancer cells). (D-F) Colony formation in cells treated with β-elemene or docetaxel at the indicated concentrations. Left: representative images of colony formation. Right: analyzed colony formation assay data. Bar graphs represent the mean ± SEM of experiments performed in triplicate.

dose of ≥ 320 μM β-elemene was required to produce the same cytotoxic effect as docetaxel (Fig. 1B, C, E, and F). However, 640 μM β-elemene was obviously toxic to MCF-10A cells (Fig. 1A and D). These results are consistent with those of previous studies in which β-elemene was toxic to tumor cells at high concentrations, but was non-toxic or minimally toxic to normal cells [2, 5, 6, 11, 12, 14, 17, 22].

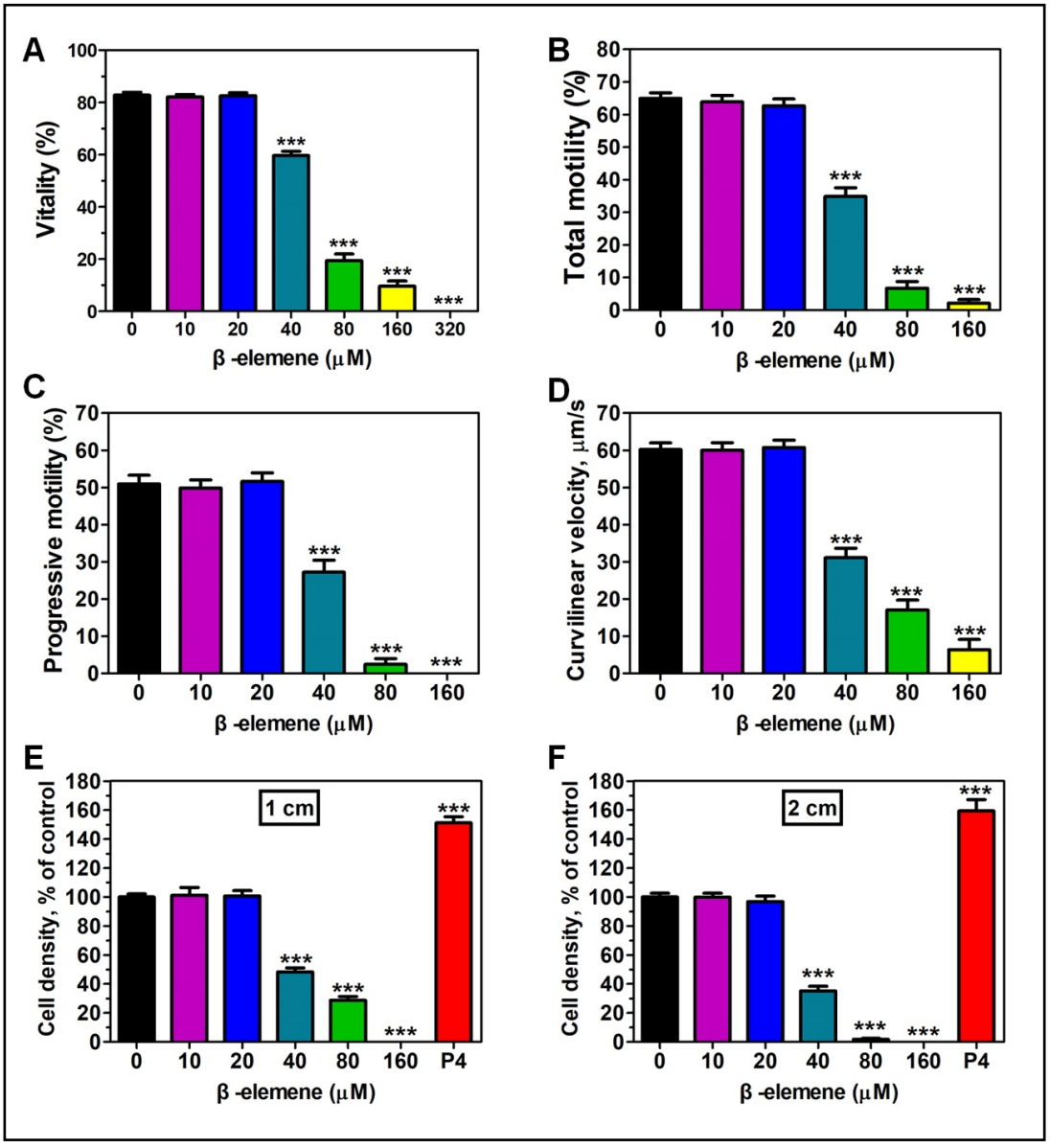

Fig. 2. Effects of β-elemene on human sperm vitality and motility in vitro. Human sperm were incubated for 2 h with different β-elemene concentrations in HTF medium at 37 °C in a 5% CO_2 incubator. (A) Sperm vitality was assessed using the eosin staining method. Total motility (B), progressive motility (C), and curvilinear velocity (D) were analyzed with the CASA system. (E and F) Assessment of sperm penetrating ability using a comprehensive evaluation of sperm motility. Sperm were first capacitated for 4 h in HTF + 25 mM $NaHCO_3$ and 0.4% HSA and then incubated with different β-elemene concentrations and 10 μM progesterone (P4, positive control). Three fields at 1 and 2 cm from the base of the tube were counted, and the average density of cells/field was calculated. Cell densities were normalized against the values for the untreated controls. Sperm from 12 individuals were analyzed for each assay. Bar: mean ± SEM. ***P<0.001, two-way ANOVA.

Subsequent examination of the cytotoxicity of β-elemene toward human sperm revealed that 40-320 μM β-elemene dose-dependently reduced human sperm vitality, and there were no viable sperm following treatment with 320 μM β-elemene (Fig. 2A). To assess whether β-elemene cytotoxicity β-elemene affects sperm function, we examined sperm motion parameters (total motility, progressive motility, and curvilinear velocity) and penetrating ability, which represented a comprehensive evaluation of sperm motility, especially hyperactivated motility. The total motility (Fig. 2B), progressive motility (Fig. 2C), curvilinear velocity (Fig. 2D), and penetrating ability (Fig. 2E and F) of human sperm decreased considerably at β-elemene concentrations of ≥ 40 μM. These results suggest that β-elemene causes injury to sperm at relatively low concentrations, which are non-toxic to cancer cells.

β-Elemene induces spontaneous acrosome reaction by increasing $[Ca^{2+}]_i$ in human sperm

Concentrations of 10 and 20 μM β-elemene did not affect human sperm vitality and motility. Thus, we assessed whether these two concentrations β-elemene affects other aspects of sperm function. Although they did not affect capacitation (Fig. 3A), 10 and 20 μM β-elemene dose-dependently enhanced the spontaneous acrosome reaction (Fig. 3B),

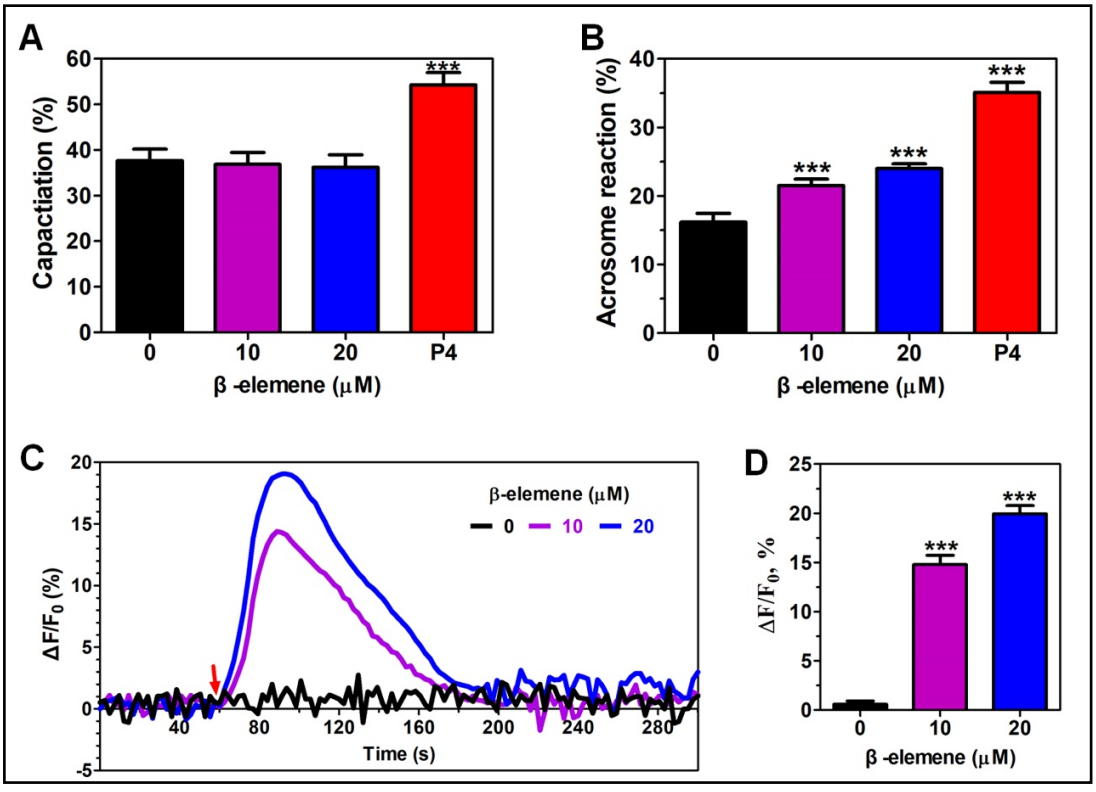

Fig. 3. Effects of β-elemene on capacitation, spontaneous acrosome reaction, and $[Ca^{2+}]_i$ of human sperm. To assess the effects of β-elemene on sperm capacitation (A), human sperm were treated for 4 h with 10 or 20 μM β-elemene and 20 μM progesterone (P4, positive control) in HTF + 25 mM $NaHCO_3$. To examine the spontaneous acrosome reaction (B), human sperm were first capacitated and then treated for 1 h with 10 or 20 μM β-elemene and 20 μM progesterone (P4, positive control). After capacitation and acrosome reaction, sperm were examined using the CTC staining method. Changes in human sperm $[Ca^{2+}]_i$ were monitored after treatment with 5 mM Fluo4-AM. Fluorescence intensity was detected using the EnSpire® Multimode Plate Reader (see Materials and Methods). Time-course curve reveals real-time changes in sperm $[Ca^{2+}]_i$ (C). Arrows indicate the timing of the addition of β-elemene to sperm samples. Inhibitory effects of β-elemene on human sperm $[Ca^{2+}]_i$ were calculated as the amplitude $\Delta F/F_0$ from a time-course curve (D). Sperm from 10 individuals were analyzed for each assay. Bar: mean ± SEM. ***P<0.001, two-way ANOVA.

which is an exocytotic process important for the penetration of the oocyte by sperm. Because the acrosome reaction is a Ca^{2+}-dependent process, we evaluated whether β-elemene affects the $[Ca^{2+}]_i$ of human sperm. Concentrations of 10 and 20 μM β-elemene initially increased human sperm $[Ca^{2+}]_i$ in a dose-dependent manner, followed by a gradual reduction in $[Ca^{2+}]_i$ to almost resting levels (Fig. 3C and D). These results indicate that β-elemene induces the spontaneous acrosome reaction by increasing $[Ca^{2+}]_i$ in human sperm.

β-Elemene affects progesterone-induced enhancement of penetrating ability and stimulation of acrosome reaction of human sperm

Progesterone facilitates fertilization by enhancing sperm function [23]. Thus, we also examined whether β-elemene affects progesterone-induced human sperm function. The ability of human spermatozoa to penetrate artificial viscous media, which mimics the viscous environment in the female reproductive tract is influenced by sperm motility, especially hyperactivated motility. The 20 μM progesterone treatment increased the mean cell numbers density by approximately 60% at 1 cm (Fig. 4A, P4) and 2 cm (Fig. 4B, P4) from the base of the tube compared with the control levels, while the addition of 10 and 20 μM β-elemene to the progesterone treatment resulted in smaller increases in cell density (P4 + 10 and P4 + 20, Fig. 4A and B). Moreover, 20 μM progesterone significantly enhanced the acrosome reaction (Fig. 4C, P4) and capacitation (Fig. 4D, P4) of human sperm. Concentrations of 10 and 20 μM β-elemene significantly inhibited the progesterone-induced acrosome reaction (P4 + 10 and P4 + 20, Fig. 4C), but did not affect progesterone-induced capacitation (P4 + 10 and P4 + 20, Fig. 4D).

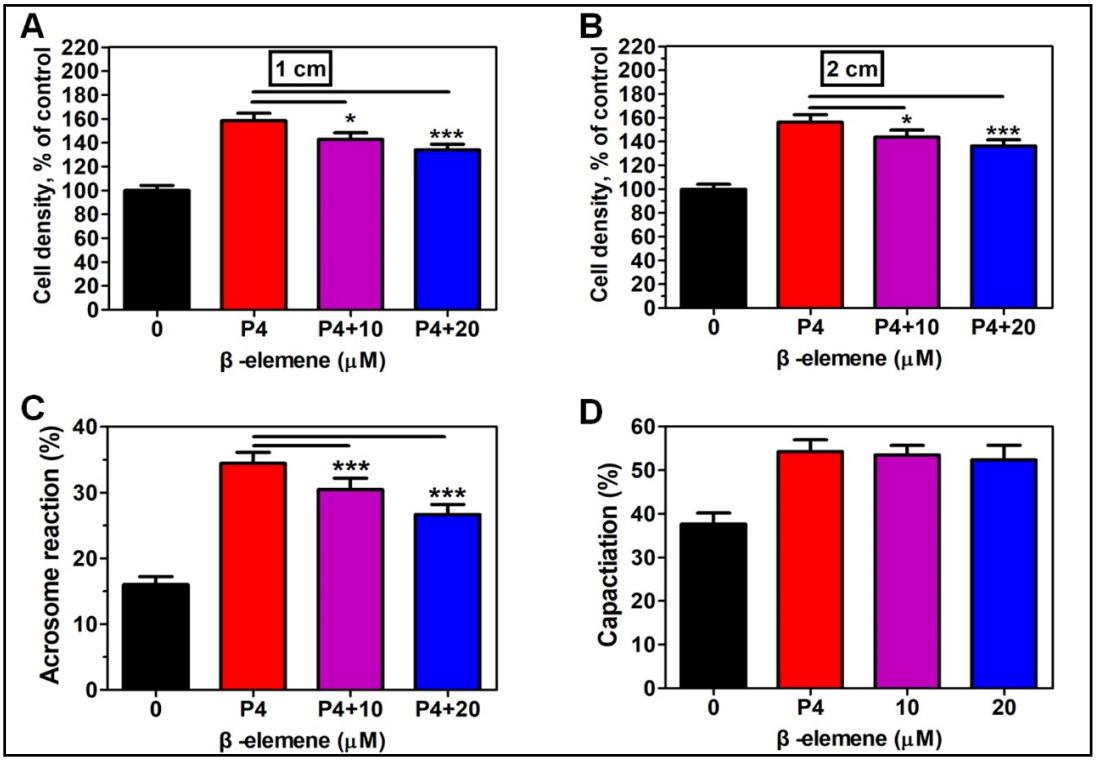

Fig. 4. Effects of β-elemene on progesterone-induced sperm functions. To evaluate the effects of β-elemene on progesterone-induced penetrating ability (A and B) and acrosome reaction (C), human sperm were first capacitated for 4 h in HTF + 25 mM $NaHCO_3$ and 0.4% HAS, and then treated for 1 h with 20 mM progesterone (P4) and different concentrations of β-elemene. To evaluate the effects of β-elemene on progesterone-induced capacitation (D), human sperm were treated for 4 h with 10 or 20 μM β-elemene and 20 μM progesterone in HTF + 25 mM $NaHCO_3$. Sperm from 10 individuals were analyzed for each assay. Bar: mean ± SEM. *P<0.05, ***P<0.001, two-way ANOVA.

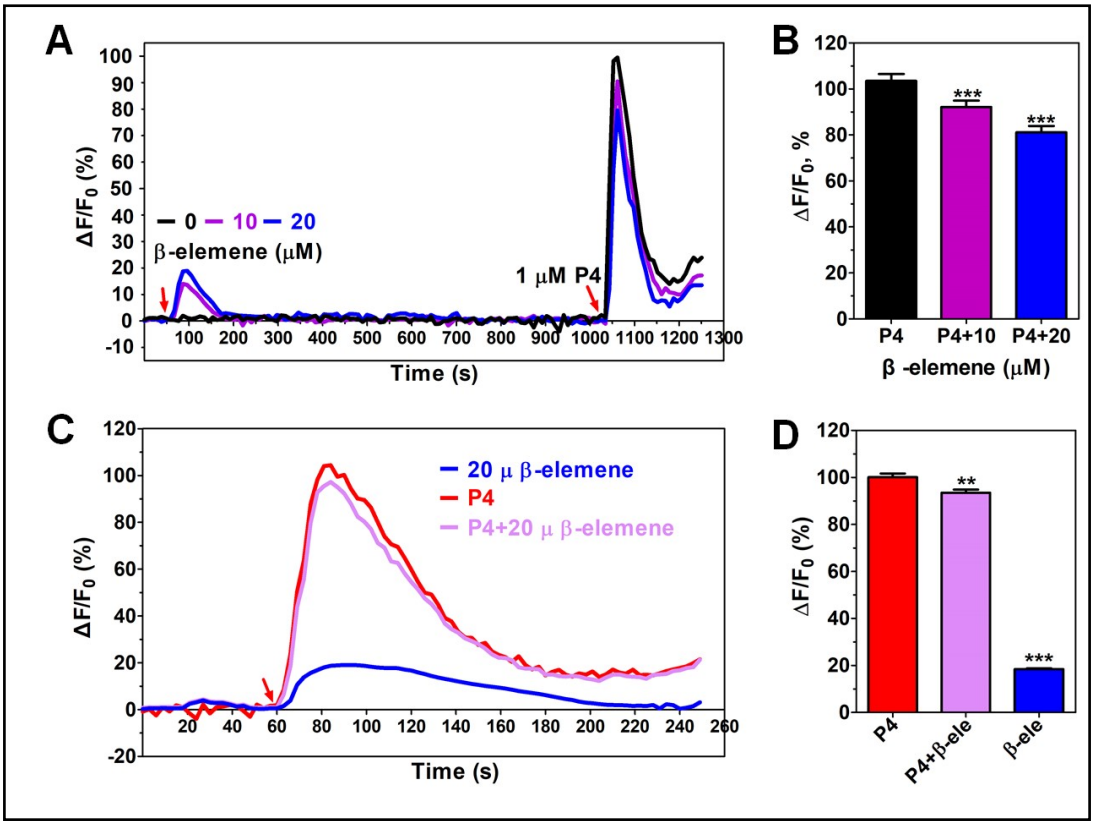

Fig. 5. Effects of β-elemene on progesterone-induced $[Ca^{2+}]_i$ increase in human sperm. (A) Human sperm were first incubated for 15 min with different concentrations of β-elemene, after which 1 μM progesterone (P4) was added. Time-course curve reveals real-time changes in sperm $[Ca^{2+}]_i$. Arrows indicate the timing of the addition of β-elemene and P4 to sperm samples. (B) Effects of β-elemene on P4-induced $[Ca^{2+}]_i$ increases were calculated as the maximum amplitude $\Delta F/F_0$ on the time-course curve. (C) Time-course curve reveals real-time changes in sperm $[Ca^{2+}]_i$ exposed to 1 μM P4 (P4), 1 μM P4 and 20 μM β-elemene (P4 + β-ele), and 20 μM β-elemene (β-ele). (D) Analysis of the real-time curve at the maximum amplitude $\Delta F/F_0$. Sperm from 10 individuals were analyzed for each assay. Bar: mean ± SEM. ***P<0.001, two-way ANOVA.

β-Elemene inhibits progesterone-stimulated Ca^{2+}influx

Progesterone-induced human sperm function is are closely correlated with progesterone-stimulated Ca^{2+} influx [23]. To clarify whether β-elemene modifies progesterone-induced Ca^{2+} signaling, we pre-treated human spermatozoa with different concentrations of β-elemene for 15 min and then added 1 μM progesterone. The increase in progesterone-induced $[Ca^{2+}]_i$ was significantly inhibited by 10 and 20 μM β-elemene (Fig. 5A and B). Furthermore, we compared the $[Ca^{2+}]_i$ amplitudes induced by β-elemene and progesterone alone or in combination. Ca^{2+} response induced by 20 μM β-elemene and 1 μM progesterone were 18.4% and 100.1%, respectively (Fig. 5C and D). Meanwhile, the Ca^{2+} response induced by the combination of β-elemene and progesterone was 93.5% (Fig. 5C and D). Lower Ca^{2+} response due to the combined treatment compared with progesterone alone was observed, suggesting that β-elemene and progesterone may use identical pathways to increase human sperm $[Ca^{2+}]_i$. These results imply that β-elemene affects progesterone-induced human sperm function by inhibiting progesterone-stimulated Ca^{2+} influx.

Discussion

β-Elemene exhibits various types of bioactivity and represents a promising compound for the development of cancer treatment. It is a liposoluble compound that can cross the blood–brain and blood–testis barriers, enabling its distribution throughout the body via blood. Also, β-elemene can accumulate in the testes at maximum concentrations of up to approximately 100 μM [24, 25]. However, whether β-elemene is toxic to the human reproductive system remains unclear. Although many phytogenic anti-tumor agents have been reported to be toxic to the human male reproductive system, several of these agents have been approved for use because most patients have no desire to preserve their fertility. However, the incidence of cancer among adolescents has recently been increasing, and thus preserving the fertility of adolescents while they undergo cancer treatment is an important concern. Consequently, the reproductive toxicity of anti-tumor agents must be thoroughly considered.

Our data indicate that 40-320 μM β-elemene is nontoxic or minimally toxic to cancer and normal somatic cells (Fig. 1), but is cytotoxic to human sperm; treatment with 320 μM β-elemene left no viable sperm (Fig. 2). These results suggest that human sperm are more sensitive to β-elemene than cancer cells or somatic cells. Thus, the cytotoxic effect of β-elemene on human sperm should be emphasized in clinical application.

Although 10 and 20 μM β-elemene treatments were not cytotoxic to human sperm, these concentrations can affect the spontaneous acrosome reaction (Fig. 3A and B), a physiological process that occurs in less than 20% of sperm after capacitation in normal males. Thus, enhancing the spontaneous acrosome reaction with β-elemene treatment may influence the penetrative ability of sperm because sperm undergo this reaction before encountering the egg. Additionally, 10 and 20 μM β-elemene treatment also inhibits progesterone-induced sperm function (Fig. 4). The acrosome reaction and progesterone-induced sperm function are Ca^{2+}-dependent processes. Therefore, we examined whether β-elemene affects sperm $[Ca^{2+}]_i$ and progesterone-induced $[Ca^{2+}]_i$ increases. Treatment with 10 and 20 μM β-elemene caused a transient increase in sperm $[Ca^{2+}]_i$, followed by a gradual decrease until reaching almost resting levels (Fig. 3C and D). Moreover, pre-incubating human sperm with β-elemene for 15 min ($[Ca^{2+}]_i$ had already decreased to almost resting levels) inhibited the progesterone-induced $[Ca^{2+}]_i$ increaseβ-elemene (Fig. 5). This discrepancy may be explained by the fact that that β-elemene and progesterone possibly use identical pathways to increase human sperm $[Ca^{2+}]_i$. During the pre-incubation of β-elemene and human sperm, the β-elemene may occupy the binding site(s) of the target protein(s) and affect the progesterone responsiveness of human sperm. This possibility is supported by the Ca^{2+} response induced by the combination of β-elemene and progesterone (Fig. 5C and D). These results imply that the non-cytotoxic β-elemene concentrations influence human sperm function *via* a Ca^{2+}-dependent mechanism.

Conclusion

The present study examined the toxicity of β-elemene toward human sperm and conclude that *in vitro* exposure to 40-320 μM β-elemene significantly inhibits sperm motility, while 10-20 μM β-elemene affects the acrosome reaction and progesterone-induced sperm function through a Ca^{2+}-dependent mechanism. This study has revealed the toxicity of β-elemene toward the human male reproductive system. Therefore, caution is required when administering β-elemene to cancer patients who wish to preserve their fertility.

Acknowledgements

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Disclosure Statement

The authors declare that they have no conflicts of interest.

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