



Research article

Effects of *Panax ginseng*, zearalenol, and estradiol on sperm functionSandra L. Gray^{1,*}, Brett R. Lackey¹, William R. Boone²¹Endocrine Physiology Laboratory, Animal and Veterinary Science Department, Clemson University, Clemson, SC, USA²ART Laboratories, Department of Obstetrics and Gynecology, Greenville Health System University Medical Group, Greenville, SC, USA

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ABSTRACT

Background: Estrogen signaling pathways are modulated by exogenous factors. *Panax ginseng* exerts multiple activities in biological systems and is classified as an adaptogen. Zearalenol is a potent mycoestrogen that may be present in herbs and crops arising from contamination or endophytic association. The goal of this study was to investigate the impact of *P. ginseng*, zearalenol and estradiol in tests on spermatozoal function.

Methods: The affinity of these compounds for estrogen receptor (ER)—alpha and beta (ER α and ER β)—was assessed in receptor binding assays. Functional tests on boar spermatozoa motility, movement and kinematic parameters were conducted using a computer-assisted sperm analyzer. Tests for capacitation, acrosome reaction (AR), and chromatin decondensation in spermatozoa were performed using microscopic analysis.

Results: Zearalenol—but not estradiol (E₂)- or ginseng-treated spermatozoa—decreased the percentage of overall, progressive, and rapid motile cells. Zearalenol also decreased spontaneous AR and increased chromatin decondensation. Ginseng decreased chromatin decondensation in response to calcium ionophore and decreased AR in response to progesterone (P₄) and ionophore.

Conclusion: Zearalenol has adverse effects on sperm motility and function by targeting multiple signaling cascades, including P₄, E₂, and calcium pathways. Ginseng protects against chromatin damage and thus may be beneficial to reproductive fitness.

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1. Introduction

The mammalian spermatozoa is a renewable cell source that provides a unique model to study the *in vitro* effects of plant extracts on cellular bioenergetics, surface membrane events, and chromatin stability. Spermatozoa motility is ultimately dependent on numerous signaling cascades, including cyclic adenosine monophosphate (cAMP) and Ca²⁺-dependent pathways [1]. Mammalian sperm undergo capacitation and the acrosome reaction (AR) prior to fertilization. Capacitation refers to a series of metabolic changes resulting in hyperactivity and eventual destabilization of surface membranes that lead to AR and ova penetration [2,3]. Decondensation of the spermatozoa nucleus is a normal event occurring during fertilization [4]. However, premature decondensation is a sign of chromatin instability and is associated with reproductive impairment [5]. Membrane and nuclear integrity can be identified by examining AR and chromatin decondensation.

Estradiol (E₂) is found in the reproductive tract fluids of males and females. E₂ seems to be required for normal germ cell development; however, exceeding this level can cause dysfunction [6,7]. Nonfertile men had higher concentrations of E₂, with 53% having seminal estradiol concentrations above the 90th percentile value for fertile men [8]. Spermatozoa are exposed to varying levels of E₂ in the male and female reproductive tract depending on various physiological and pathophysiological factors. With the identification of aromatase in spermatozoa, coupled with up to 10-fold higher levels of testosterone in seminal fluid, the exact level of exposure to E₂ is difficult to accurately determine for an intracrine mechanism [9]. The seminal plasma of breeding boars had 92 pg/mL E₂ and the seminal plasma of bulls was found to contain 568 pg/mL [10]. Concentrations of E₂ range from 8 to 300 ng/mL in sows [11] and up to 2,295 ng/mL in mature human ovarian follicles [12]. During *in vitro* maturation of oocytes, E₂ concentrations of 1–10 μ g/mL have been used in culture media [13–15].

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Concentrations of E_2 at 0.1 $\mu\text{g}/\text{mL}$ stimulated the forward migration of ejaculated human, but higher concentrations were no different from controls [16]. E_2 increased motility, oxidative metabolism, and intracellular ATP, and exhibited higher progressive velocity, linear motility, and longevity in normal and asthenozoospermic patients [17–19]. The binding of spermatozoa to oviductal epithelial cells favors spermatozoa survival and capacitation. Neither attachment to oviductal cells nor AR in boar spermatozoa was affected by E_2 [20]. However, the ability of oviductal epithelium to prolong the motility of bovine spermatozoa was enhanced by E_2 [21].

Panax ginseng has been used for thousands of years as an adaptogen, to increase physical energy and stamina and to enhance fertility. In clinical studies of oligospermic patients, ginseng was able to increase spermatozoa motility and number [22], perhaps by modulating nitric oxide (NO) formation [23]. Numerous reports of estrogen-like activity in men and women after ginseng use have been published, but data supporting the mechanism are unclear [24–27]. Clinical studies on oligospermic patients suggested that ginseng increased sperm motility and number [22,28]. The effects of ginseng extract on chromatin decondensation have not been reported.

The identification of chemical constituents in ginseng with pharmacological activity first occurred in 1950s and 1960s [29]. These compounds were identified as triterpene saponins and named ginsenosides Rx from “a” to “s” according to their mobility on thin layer chromatography. More than 30 different glycoside ginsenosides have been isolated from ginseng roots [30,31].

In a prior study, it was hypothesized that ginseng, like most plants with phytoestrogenic compounds, would display preference for estrogen receptor (ER) β over ER α [32,33]. When two of the main ginsenosides (Rg1 and Rb1) did not account for the appreciable binding to ER α or ER β , further investigation revealed that a significant amount of the activity in the tested ginseng roots originated from zearalenone and its metabolite α -zearalenol, which are mycoestrogens produced by *Fusarium* fungus [34].

Zearalenone has been identified as a contaminant in food and agricultural commodities worldwide [35,36]. Grains and corn foods in Canada were analyzed for zearalenone between 1986 and 1993. Concentrations of the mycotoxin ranged from 23 to 215 ng/g [37]. Studies of stored wheat and grain samples from China, Korea, Brazil, and Wisconsin (USA) have also shown zearalenone contamination [30,38–41].

The effect of these mycoestrogens on human health is unclear. The toxic effects on liver, kidney, immune, reproductive, and fetal outcomes in addition to carcinogenicity are mostly known from experimental models. Extrapolation to humans may not be accurate because of inadequate food consumption data, lack of knowledge about relative health risks, and the possibility of synergism with other mycotoxins present in the same food commodities [42]. Current reporting probably underestimates the effect of mycotoxins as a cause of human mortality [43,44].

Zearalenone is rapidly absorbed following oral administration and quickly transformed into α - and β -zearalenol by 3α -hydroxysteroid dehydrogenase in the liver. These metabolites are more potent than zearalenone, with the estrogenic activity of α -zearalenol about three to four times higher than β -zearalenol and are recognized as a source of toxicity in farm animals [45–47]. Zearalenone ingestion through contaminated feed is associated with decreased reproductive capacity and hyperestrogenic conditions such as vaginal swelling, enlargement of mammary glands, and testicular atrophy [46]. Purified zearalenone fed to groups of healthy, multiparous sows produced multiple reproductive deficiencies including infertility, constant estrus, pseudopregnancy, and diminished fertility [48]. Spontaneous abortions in a herd of cattle were also linked to zearalenone-contaminated hay [48,49]. A single dose of zearalenone induced

testicular germ cell apoptosis in rats in a time-dependent and stage-specific pattern resulting in germ cell depletion and testicular atrophy [38]. Zearalenone is also cytotoxic to male mice [50].

The purpose of this study is to elucidate the effects of ginseng on spermatozoal function along with estradiol and the fungal metabolite, zearalenol. By using sperm function tests in addition to binding assays for ER α and ER β , we hope to provide more understanding of the regulatory events governing physiological function. The influence of E_2 , ginseng, and α -zearalenol on sperm energetics, membrane events, and chromatin stability will be presented.

2. Materials and methods

2.1. Plant materials for sperm function studies

Dried roots from 5-year-old ginseng plants were obtained from commercial farms in Illinois and Indiana, USA, and then powdered using a grinder and stored separately in air-tight glass containers at 4°C. Crude extracts of 1-g samples used for the competitive binding assays were extracted by using 10 mL of either deionized water (initially at 100°C) or 80% methanol (Mallinkrodt Nanograde; VWR, Atlanta, GA, USA). Samples were extracted with solvent for approximately 8 h on a lateral shaker at room temperature, centrifuged at 1,800g for 15 min, and the supernatant removed. The pellet was resuspended in 8 mL solvent and mixed overnight. Supernatants were combined, placed in a Thermolyne Dri-Bath (VWR) at 40°C, and evaporated to dryness with filtered air. Samples were resuspended in 1 mL ethanol and filtered using 0.45- μm polyvinylidene fluoride (PVDF) Acrodisc (Pall Gelman, Ann Arbor, MI, USA). All dried samples were extracted and assayed three times.

2.2. Preparation and treatment of boar spermatozoa

Duroc semen from Lean Value Sires (New Carlisle, OH, USA) was collected from boars with high fertility ratings, filtered, and then diluted in Mulberry (Swine Genetics International; Cambridge, IA) commercial long-term storage extender. Semen was packaged so the temperature was maintained at approximately 17°C and shipped overnight.

Sperm preparation methods using nontoxic plasticware were adapted from *Practical Laboratory Andrology* [51,52]. The extended semen from six boars was pooled and centrifuged at 400g for 5 min to remove the extender. Spermatozoa samples were washed twice with 0.2mM NaHCO₃ Tyrode's albumin–lactate–pyruvate medium, and motile spermatozoa were separated using the swim-up method [52]. The supernatants containing the motile spermatozoa were pooled and diluted in media to obtain a concentration of $2\text{--}3 \times 10^7$ spermatozoa/mL. One-milliliter aliquots of the diluted spermatozoa were used for each treatment.

Final concentrations in the samples tested were as follows: (1) E_2 , 10 $\mu\text{g}/\text{mL}$; (2) ginseng extract, 2 mg/mL; and (3) α -zearalenol, 10 $\mu\text{g}/\text{mL}$ [53]. Extracts of ginseng were prepared by adding 10 mL 80% methanol to 2 g powdered root samples and shaking for 5 h. Samples were centrifuged at 1,800g for 15 min and the supernatants removed. The extraction was repeated, then supernatants were combined and evaporated to dryness under a gentle stream of filtered air. Dried samples were reconstituted to 1 g/mL 100% ethanol and filtered using 0.45 μm PVDF and stored at 4°C. Stock solutions for E_2 and α -zearalenol were prepared at 1 mg/mL in 100% ethanol and further diluted with ethanol as needed.

2.3. Spermatozoa motility analysis

One-milliliter aliquots of the diluted motile spermatozoa were added to 12 \times 75 mm polypropylene tubes that contained test

compounds that had been evaporated to dryness. Motion parameters and kinematics were evaluated at 0, 30, 60, 120, 180, 240, and 720 min for each treatment using μ Cell slides (20 μ m; Conception Technologies, La Jolla, CA, USA) on a Hamilton Thorne Version 12 Integrated Visual Optical (IVOS) Spermatozoa Analysis System (Hamilton Thorne Research, Beverly, MA, USA). Samples were assayed in triplicate with a minimum of 200 spermatozoa cells analyzed for each replicate (Fig. 1, Table 1). Data from two separate experiments were collected using spermatozoa from two shipments.

2.4. Spermatozoa capacitation, AR, and chromatin decondensation

Swim-up spermatozoa ($2\text{--}3 \times 10^7/\text{mL}$) were incubated under capacitating conditions using 25mM NaHCO_3 Tyrode's albumin–lactate–pyruvate buffer and incubating in 5% CO_2 at 37°C for 180 min. Test compounds were prepared as described for the motility experiments. Calcium ionophore A23187 (Cat. No. C7522; Sigma Chemical; St Louis, MO, USA) and progesterone (P_4) (Cat. No. 3972; Sigma Chemical) were added at concentrations of 2.5 μ M and 100nM, respectively, to aid in initiation of AR. After 180 min, samples were removed from the incubator and slides were prepared for evaluation of AR and chromatin decondensation. Slides were prepared in duplicate for AR staining [54]. Spermatozoa

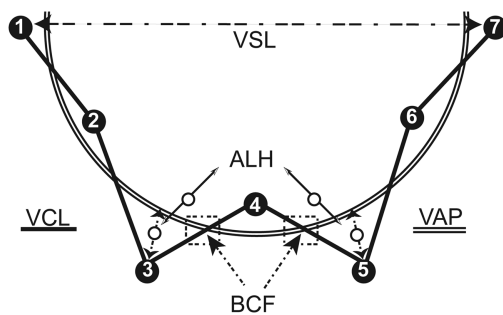


Fig. 1. Diagram of computer-assisted sperm analyzer (CASA) kinematic parameters. 1–7 represent path of the spermatozoal head.

Table 1
Definitions of CASA terms

Motility	% of sperm cells that are motile defined by VAP > 10 $\mu\text{m/s}$
Progressive motility	% of motile cells with VAP > 45 $\mu\text{m/s}$ and LIN > 45%
Rapid	% of motile cells with VSL > 75 $\mu\text{m/s}$
VCL	Time-averaged velocity of the sperm head along its actual trajectory
ALH	Amplitude of variations of the actual sperm head displacement about its average trajectory
VSL	Time-average velocity of the sperm head along a straight line from (VSL) its first position to its last position
VAP	Time-average velocity of the sperm head along its average trajectory
LIN	Linearity of the curvilinear velocity (VCL/VAP)
STR	Straightness of the average path (VSL/VAP)
BCF	Time-average rate at which the actual sperm trajectory crosses the average path trajectory.

ALH, amplitude of lateral head displacement; BCF, beat cross frequency; CASA, computer-assisted sperm analyzer; LIN, linearity; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity.

(30 μL) were smeared and allowed to air-dry on a microscope slide for 15–30 min. A 10-min fixation of slides was accomplished with 100% methanol. Fixed spermatozoa were then exposed to 30 μL of FITC-labeled peanut agglutinin (FITC-PNA; Cat. No. L-7381; Sigma Chemical) and placed in a moist, dark chamber at 4°C for 30 min. The slides were rinsed twice with phosphate-buffered saline (PBS) and once with distilled water and in order to preserve fluorescence, then immediately treated with 10 μL antifade solution (Cat. No. P-7481; Molecular Probes; Eugene, Oregon), and a coverslip was applied. Slides were examined using a Nikon (Optipho 2; Nikon Inc., Melville, NY, USA) microscope equipped with epifluorescent illuminator at excitation wavelength 450–490 nm. Acrosome status was determined for 200 sperm from each slide.

For determination of chromatin decondensation, duplicate slides were prepared for each treatment [55–57]. Aliquots of 25 μL were spread and allowed to air-dry on microscope slides followed by fixation in 3% (v/v) glutaraldehyde (Tousimis Research Corp., Rockville, MD, USA) in PBS for 30 min at room temperature. A 5% solution of aniline blue was diluted in PBS, adjusted to pH 3.5 and filtered using Whatman No. 2 filter paper (VWR). Slides were stained for 5 min at room temperature, and then rinsed twice with PBS and once with distilled water. Slides were air-dried and coverslips were mounted with Cytoseal 60 (VWR). Cells were considered normal if aniline blue stain was not taken up by the nucleus; blue-stained heads represented chromatin decondensation. Slides were examined as described above except that bright-field illumination was used. Data were analyzed for statistical differences using analysis of variance (SAS Institute, Cary, NC, USA).

2.5. Plant materials for ER binding assay

Korean and Red ginseng were a generous gift from Dr J.L. McLaughlin (Nature's Sunshine Products, Inc., Spanish Fork, UT, USA). Plant material was extracted at Nature's Sunshine Products by sonicating approximately 10 g ground plant material (W_1) with 100 mL ethyl acetate for 2 h and with occasional shaking, then filtering through Whatman No. 1 filter paper. The extraction was repeated again on the pellet and filtrates were combined. The filtrate was evaporated to approximately 1–3 mL under a steady stream of N_2 , transferred to a 5-mL bottle, and evaporated to dryness. The dried extract was weighed (W_2), then the percentage of extract from the sample was calculated ($W_1/W_2 \times 100$) and shipped to the Endocrine Physiology Laboratory at Clemson University (Clemson, SC, USA) for analysis.

Dried extracts from Nature's Sunshine equivalent to 2 g of original ground plant were suspended overnight in 3 mL hexane. Three milliliters of 80% methanol were added to the hexane plant suspension, and the mixture shaken for 1 h. Samples were centrifuged at 1,800g for 15 min. The hexane supernatant was pipetted into another tube and extracted a second time with 80% methanol. The two methanol fractions were combined, filtered by using 0.45- μm PVDF membrane (Acrodisc; Pall Gelman, Ann Arbor, MI, USA) and evaporated to dryness on a heated DriBath (45°C) under a gentle stream of filtered air. Dried plant extracts were reconstituted to a concentration of 1 g of original plant material per mL in 100% ethanol.

2.6. ER binding assay

All chemicals were obtained from VWR, Fisher (Atlanta, GA, USA) or Sigma Chemical unless otherwise noted. Estradiol [$2,4,6,7\text{-}^3\text{H}(\text{N})$] (71 Ci/mmol) was purchased from NEN Life Sciences Products (Boston, MA, USA). Human recombinant ER α and ER β were obtained from PanVera (Madison, WI, USA). Standard concentrations (diluted in 100% ethanol) and plant extracts were added to assay tubes, evaporated to dryness under filtered air,

reconstituted in 50 μ l assay buffer, and then processed according to a validated protocol [34]. A four-parameter logistic standard curve was constructed using StatLIA Analysis software (Brendan Scientific; Carlsbad, CA, USA). The concentration of test samples displacing 50% of $^3\text{H-E}_2$ binding from each receptor (IC_{50}) was determined from the standard curve, and the relative binding affinity was expressed as estrogen binding equivalents. Data were analyzed for statistical differences using analysis of variance (SAS Institute).

3. Results

3.1. Motility analysis of spermatozoa treated with E_2 , ginseng, and α -zearalenol

E_2 and ginseng extract added to high-fertility boar spermatozoa had no significant effect on overall motility, progressive motility, or rapid motility percentages when compared to controls, whereas extracts of α -zearalenol significantly inhibited all three motilities of these spermatozoa (Table 2). Motility patterns for E_2 and ginseng

Table 2

Effects of estradiol, ginseng extract, and α -zearalenol on boar spermatozoa movement parameters

Treatment	Spermatozoa movement parameters		
	Motility (%)	Progressive motility (%)	Rapid motility (%)
Control	83.5 \pm 6.4 ^a	52.7 \pm 52.7 ^a	62.0 \pm 10.5 ^a
Estradiol	84.7 \pm 6.2 ^a	53.9 \pm 13.0 ^a	63.8 \pm 12.0 ^a
Ginseng	82.1 \pm 6.6 ^a	48.8 \pm 12.7 ^a	57.8 \pm 12.0 ^a
α -Zearalenol	30.2 \pm 18.8 ^b	11.9 \pm 12.3 ^b	15.4 \pm 14.4 ^b

^{a,b} Numbers represent the overall mean \pm SD of measurements at 0, 30, 60, 120, and 240 min.

Numbers with different superscripts within each spermatozoa movement parameter are significantly different from each other ($p < 0.05$). Concentrations used for estradiol, ginseng, and α -zearalenol were 10 $\mu\text{g}/\text{mL}$, 2 mg/mL , and 10 $\mu\text{g}/\text{mL}$, respectively. $N = 30$ for estradiol, ginseng, and α -zearalenol; $N = 32$ for control.

treatments at time points 30, 60, 120, 180, 240, and 720 min were similar to those of control samples (Figs. 2A–2C). The percentage motile cells did not decrease from the levels at time 0 for control, E_2 , and ginseng treatments until after the 240-min time point. Control and E_2 -treated samples decreased below time 0 in percentage progressive and rapid motile cells by 240 min ($p = 0.05$), whereas ginseng-treated samples had a decline by 180 min. For α -zearalenol, the overall percentage of motile, progressive, and rapid cells significantly decreased below control levels at time 0 within 30 min of treatment.

There were no differences in mean values over the time trial for kinematic parameters between control, E_2 , and ginseng treatments (Table 3). Likewise, the patterns over time for E_2 and ginseng were similar to those of control (Figs. 2D–2F). The curvilinear velocity (VCL) decreased below time 0 values for the control, E_2 -, and ginseng-treated samples at 240 min. There were no changes in amplitude of lateral head displacement (ALH) or linearity (LIN) values between time 0 and 240 min for control, E_2 , or ginseng treatments. The patterns were different, however, for α -zearalenol treated cells; in these cells, VCL decreased at 30 min, ALH at 120 min, and LIN at 120 min.

3.2. Effects of E_2 , ginseng, and α -zearalenol on spermatozoa AR

Ginseng and α -zearalenol treatments inhibited spontaneous AR when compared to control or E_2 (Table 4). Overall, ionophore-treated cells dramatically increased the percentage AR above the nontreated cells. When cells were incubated with ionophore, the percentage of AR spermatozoa treated with E_2 or ginseng did not differ significantly from that of the control, but α -zearalenol treatment resulted in a significantly greater percentage of AR spermatozoa. For sperm incubated with P_4 only, no changes in rates of AR were observed. In spermatozoa incubated with ionophore and P_4 , there was a significantly lower percentage of acrosome reacted spermatozoa following ginseng treatment and significantly

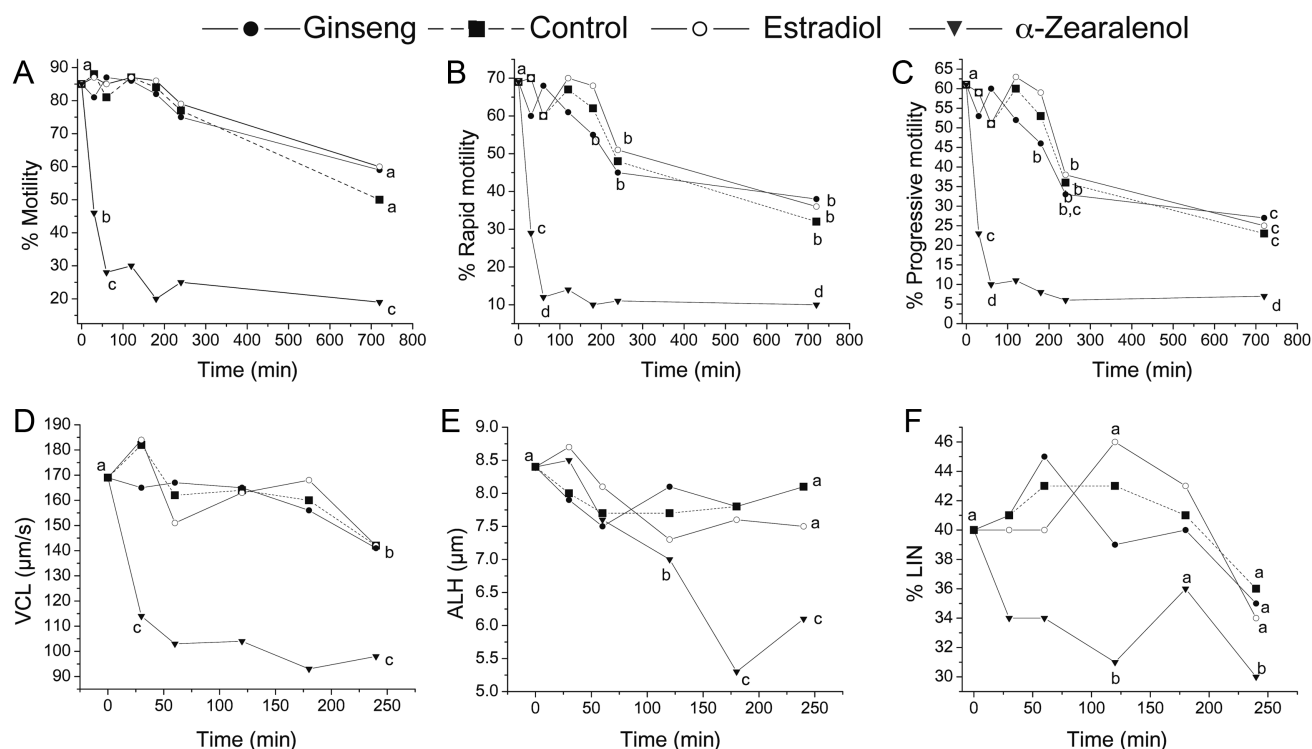


Fig. 2. Effects of ginseng, estradiol, and α -zearalenol on boar spermatozoa. (A) Percent motility. (B) Rapid motility. (C) Progressive motility. (D) VCL kinematics. (E) ALH kinematics. (F) Percent LIN. Letters denote significant difference ($p < 0.05$). ALH, amplitude of lateral head; LIN, linearity; VCL, curvilinear velocity.

Table 3Effects of estradiol, ginseng extract, and α -zearalenol on boar spermatozoa kinematic parameters

Treatment	Spermatozoa kinematic parameters			
	VAP ($\mu\text{m/s}$)	VSL ($\mu\text{m/s}$)	VCL ($\mu\text{m/s}$)	ALH ($\mu\text{m/s}$)
Control	85.2 \pm 16.5 ^a	64.0 \pm 14.0 ^a	161.4 \pm 29.7 ^a	7.9 \pm 1.0 ^a
Estradiol	87.2 \pm 14.9 ^a	65.4 \pm 14.2 ^a	161.8 \pm 24.3 ^a	7.8 \pm 1.0 ^a
Ginseng	82.8 \pm 16.5 ^a	62.8 \pm 14.7 ^a	158.8 \pm 28.1 ^a	7.8 \pm 1.0 ^a
α -Zearalenol	49.8 \pm 10.4 ^b	31.5 \pm 7.7 ^b	102.8 \pm 19.0 ^b	7.0 \pm 2.1 ^b

Treatment	BCF (Hz)	STR (%)	LIN (%)
Control	33.5 \pm 2.2 ^a	71.1 \pm 4.8	40.6 \pm 4.8 ^a
Estradiol	32.8 \pm 2.8 ^a	71.0 \pm 5.4	38.8 \pm 6.5 ^a
Ginseng	32.5 \pm 3.4 ^a	71.5 \pm 5.6 ^a	40.5 \pm 5.6 ^a
α -Zearalenol	31.7 \pm 5.6 ^b	62.8 \pm 6.5 ^b	33.1 \pm 5.3 ^b

^{a,b} Numbers represent overall mean \pm SD of parameters at 0, 30, 60, 120, and 240 min. Numbers with different superscripts are significantly different from each other ($p < 0.05$). Concentrations used for estradiol, ginseng extract, and α -zearalenol were 10 $\mu\text{g/mL}$, 2 mg/mL, and 10 $\mu\text{g/mL}$, respectively. $n = 30$ for estradiol and ginseng; $n = 28$ for α -zearalenol; $n = 32$ for control. ALH, amplitude of lateral head displacement; BCF, beat cross frequency; LIN, linearity; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity.

higher percentage of AR spermatozoa following α -zearalenol treatment. The increased percentage of AR spermatozoa seen with ionophore-treated alone (90%) was reduced to 74.2% when P_4 was added with the ionophore.

3.3. Effects of E_2 , ginseng, and α -zearalenol on spermatozoa chromatin decondensation

The percentage spermatozoa exhibiting decondensation for E_2 - and ginseng-treated samples were similar to control, but α -zearalenol-treated samples were significantly higher (Table 4). Ginseng-treated spermatozoa in the presence of ionophore had significantly less decondensation than ionophore-treated control, and E_2 -treated and α -zearalenol-treated spermatozoa samples.

Table 4

Acrosome reaction and chromatin decondensation in boar spermatozoa

Treatment	Subtreatment	Acrosome reacted (% \pm SD)	Decondensation (% \pm SD)
Control	None	9.0 \pm 6.2 ^a	9.4 \pm 5.0 ^a
Estradiol	None	7.8 \pm 2.5 ^a	19.1 \pm 7.0 ^a
Ginseng	None	2.5 \pm 1.2 ^b	22.1 \pm 11.7 ^a
α -Zearalenol	None	3.5 \pm 1.0 ^b	51.8 \pm 16.0 ^b
Control	Iono	70.3 \pm 6.8 ^a	93.7 \pm 4.3 ^a
Estradiol	Iono	57.8 \pm 4.3 ^a	94.7 \pm 3.2 ^a
Ginseng	Iono	64.8 \pm 22.0 ^a	72.5 \pm 6.2 ^b
α -Zearalenol	Iono	90.0 \pm 2.4 ^b	97.5 \pm 2.4 ^a
Control	P_4	4.8 \pm 2.0 ^a	8.25 \pm 6.1 ^a
Estradiol	P_4	6.5 \pm 0.3 ^a	25.2 \pm 6.5 ^b
Ginseng	P_4	5.0 \pm 0.8 ^a	17.5 \pm 7.2 ^b
α -Zearalenol	P_4	7.0 \pm 3.7 ^a	98.0 \pm 16.0 ^c
Control	Iono + P_4	60.9 \pm 12.7 ^b	91.5 \pm 3.7 ^a
Estradiol	Iono + P_4	55.9 \pm 7.3 ^b	91.5 \pm 3.4 ^a
Ginseng	Iono + P_4	32.3 \pm 7.1 ^a	93.7 \pm 4.0 ^a
α -Zearalenol	Iono + P_4	74.2 \pm 5.8 ^c	97.5 \pm 1.3 ^a

^{a,b,c} Values with different superscripts are significantly different ($p < 0.05$) within each subtreatment group. Percent of acrosome reacted is based on a total of 400 cells counted from two different cell populations. Concentrations used for estradiol, ginseng extract, and α -zearalenol were 10 $\mu\text{g/mL}$, 2 mg/mL, and 10 $\mu\text{g/mL}$, respectively. Iono (calcium ionophore A23187) was used at 2.5 μM and P_4 (progesterone) was used at 100nM. Formula for chromatin decondensation = (aniline-blue staining cells \div total cells).

Decondensation rates for E_2 -, ginseng-, and α -zearalenol-treated spermatozoa were higher than that of control with P_4 addition to the treatments. All samples with ionophore and P_4 addition had greater than 90% decondensation.

3.4. ER binding assay

Korean and Red ginseng roots displayed several-fold higher binding for ER β than for ER α ; however, the binding of α -zearalenol was much higher than that in both ginseng samples and slightly higher than that of E_2 for each of the ERs (Table 5).

4. Discussion

Spermatozoa are unique, motile cells that provide sensitive endpoints for evaluating the impact of plant extracts on cellular function. Spermatozoa motility, AR, and chromatin decondensation are endpoints regulated by intrinsic and extrinsic factors. Spermatozoa under *in vitro* capacitating conditions may exhibit increases in the percent of cells showing motility and show changes in kinematic parameters resulting in hyperactive patterns of motility. This state of hyperactivity in the spermatozoa is associated with increased ALH movement and VCL, which result in increased flagellar movement. Another characteristic of hyperactivity is decreased LIN [52]. Flagellar activity is initiated and regulated by PKA and other protein kinases, which initiate a cascade resulting in phosphorylation of proteins associated with movement. Hydrolysis of mitochondrial ATP drives microtubule sliding, resulting in flagellar movement. Altered wave movement and direction are regulated by concentrations of cellular Ca^{2+} regulated kinase cascades [58–60]. The AR is affected by ionic regulators such as Ca^{2+} , H^+ , K^+ , and Na^+ as well as the hormones P_4 and E_2 . Compounds that interfere with or stimulate these factors may influence AR. Chromatin in the mature spermatozoa is tightly condensed, and peroxidative damage from agents in the external environment may cause unwinding or decondensation of the chromatin [61].

4.1. Effects of E_2 , ginseng, and α -zearalenol on spermatozoa motility

The mean percentage of overall, progressive, and rapid motile cells including time points at 0, 30, 60, 120, 180, and 240 min were not different for control, E_2 -treated, and ginseng-treated samples. Similarly, the mean kinematic factors were not different. However, α -zearalenol inhibited motility threefold over the control or other treated samples in spermatozoa from animals with high fertility status. The mechanism for the observed dramatic decrease in spermatozoa motility is uncertain, but may involve α -zearalenol-mediated inhibition of mitochondrial ATPase/ATP synthase [62]. ATP is integral to spermatozoa motility, and any compound that interferes with its production could decrease motility. Fertilization may also be impaired by zearalenone and α -zearalenol inhibiting the ability of boar spermatozoa to successfully bind to the zona pellucid [63,64].

Table 5Relative binding affinity of plant extracts for estrogen receptor (ER) α and ER β

Extracts or compounds	N	ER α	ER β
Korean ginseng root	6	$< 1 \times 10^{-7}$	1.9×10^{-6}
Red ginseng root	6	2.5×10^{-7}	1.8×10^{-5}
α -Zearalenol	6	111	125
Estradiol-17 β (E_2)	6	100	100

Relative binding affinity (RBA) = concentration (μg) of estrogen receptor binding equivalents (EBE)/gram test sample as determined from an E_2 standard curve divided by the EBE for $E_2 \times 100$, where E_2 is assigned an arbitrary value of 100.

In addition to adversely affecting functionally competent spermatozoa, zearalenone included in the diet of rats induced testicular hypoplasia of the germinal epithelium in rats and prevented production of mature spermatozoa [65]. Apoptosis is thought to be the principal mechanism contributing to germ cell depletion and testicular atrophy following zearalenone exposure [38].

An ER has been identified on the membrane of human spermatozoa membrane. Binding of E₂ to this receptor induces a rapid increase in intracellular Ca²⁺ concentration but interfered with P₄ effects on spermatozoa [66,67].

Additionally, E₂ produced an increase in tyrosine phosphorylation during capacitation. Whereas some studies report significant increases in human spermatozoa motility with E₂ treatment *in vivo*, others report no difference from control [17,68,69]. When compared to controls, E₂ treatment tended to increase motility parameters, although not significantly, in boar spermatozoa. The discrepancy among these results may be attributable to the fertilizing competency of the spermatozoa. A preliminary unpublished study by the authors indicated that sperm from less-fertile boars were less responsive to the actions of zearalenol.

Unlike a study that reported increases in motility *in vitro* from human spermatozoa of inferior quality with *Panax notoginseng* extract *in vitro* treatment [70,71], treatment with *P. ginseng* root extract did not significantly improve the mean motility parameters above the control levels in spermatozoa from boars with high fertility status. The stimulation of motility by the *P. notoginseng* extract was attributed to the ginsenoside Rc and, to a lesser, degree Rb2 [70]. Concentrations of these ginsenosides in the extract in this study were not quantified and may not have been present in sufficient quantities to stimulate motility in the same manner. There were no differences in the kinematic parameters VCL, ALH, and LIN for control, E₂, and ginseng treatment, suggesting that these compounds did not positively or negatively influence the cellular energetics or ion flux.

4.2. Effects of E₂, ginseng, and α -zearalenol on spermatozoa AR

Spontaneous AR in boar spermatozoa was significantly less after treatment with ginseng and α -zearalenol. AR is dependent on intracellular Ca²⁺ and Na⁺ concentrations, and ginseng has been shown to suppress Ca²⁺ and Na⁺ channel currents in a dose-dependent manner [72,73]. AR is preceded by phospholipase activation [74], and ginseng has been shown to inhibit phospholipase activity [75,76]. Ginseng and zearalenone may modulate some of the protein kinases involved in AR [77–80]. Adding a calcium ionophore to samples enabled the spermatozoa to overcome the cellular changes induced by ginseng and α -zearalenol, which inhibited AR. Although AR was not induced in ginseng-, E₂-, or α -zearalenol-treated spermatozoa with the addition of P₄ alone, rates of AR with P₄ and ionophore were induced in the samples in a similar manner as with ionophore alone. Ginseng-treated spermatozoa with P₄ and ionophore had a 50% reduction in AR reaction when compared to control spermatozoa with ionophore alone. This inhibition may be related to P₄ interactions with phospholipase A2 and gamma-aminobutyric acid (GABA) receptor pathways. P₄ leads to activation of phospholipase A2 in spermatozoa, which, in turn, stimulates AR [81,82]. *P. notoginseng* was shown to inhibit phospholipase A2 activity [83], and perhaps *P. ginseng* has a similar activity, preventing the P₄ activation of phospholipase and delaying AR [84]. Progesterone interacts with the GABA receptor located on the spermatozoa membrane and promotes hyperactivity and AR [85–87]. Ginsenosides Rg1 and Rb1, the major ginsenosides of *P. ginseng*, were shown to bind GABA receptors [88]. Ginseng extract may modulate with P₄ and ionophore pathways that promote AR.

One potential mechanism that may account for this interaction is crosstalk between NO/cGMP/PKG and ER β receptor cascades with PKB/Akt being a likely intermediary [89–95]. Ginseng has been shown to stimulate NO formation in mammalian cells, and ginsenoside Re increases human sperm capacitation and AR through a NO/cGMP/PKG pathway [23,96]. Evidence of another estrogen signaling pathway in spermatozoa involving a membrane estrogen receptor (MER; also known as GPR30) has been accumulating [97]. The presence of these systems within spermatozoa suggests that estrogenic and xenoestrogenic compounds may directly or indirectly affect spermatozoa function by modulating cAMP (PKA), Ca²⁺ (PKC), or general kinase activity [97–99].

4.3. Effects of E₂, ginseng, and α -zearalenol on spermatozoa chromatin decondensation

Chromatin decondensation in test samples was stimulated significantly above control only by α -zearalenol treatment. Similar findings were reported in horses and boars exposed to zearalenone and α -zearalenol [100,101]. Cattle implanted with Zeranone, a synthetic anabolic steroid derived from zearalenone, also showed altered chromatin structure of spermatozoon [101,102]. Chromatin abnormalities were also observed in bovine oocytes exposed to zearalenone and α -zearalenol [103]. Whereas all samples showed considerable rates of chromatin decondensation with addition of ionophore, only ginseng-treated samples had significantly less decondensation than the control. Ginseng is an antioxidant and may serve as a recipient for the reducing equivalents required to maintain chromatin structure [104].

E₂- and ginseng-treated samples with P₄ had slight but significant increases in chromatin unwinding when compared to control with P₄. However, when compared to decondensation rates without P₄ addition, there were no differences between E₂- and ginseng-treated samples. Neither ginseng nor E₂ worked synergistically with P₄ to increase decondensation rates. Perhaps P₄ dampens decondensation responses to ginseng extract and E₂. There is evidence that E₂ interferes with P₄ action in human spermatozoa, including an inhibition of P₄-stimulated calcium flux [66,67]. Pearce et al [105] showed that ginseng extract bound the P₄ receptor in rat uterine tissue. By contrast, α -zearalenol proved to be a powerful chromatin decondensation agent with P₄, perhaps compromising the disulfide bonds required for chromatin condensation.

Using boar spermatozoa as model, the physiological events of motility, AR and chromatin decondensation were examined using plant extracts that were screened for compounds that could influence cellular energetics, membrane stability, and chromatin damage. *In vitro* tests can also be used to evaluate the positive or negative impact a plant or its compounds might have on male fertility. From these experiments, it was concluded that α -zearalenol significantly reduced spermatozoa motility parameters and, thus, may interfere with cAMP production and mitochondrial function. Additionally, α -zearalenol treatment was damaging to chromatin structure in all treatments, suggesting that it may have altered cellular redox levels including cofactor NADPH. E₂ showed a positive trend, although not statistically significant, on motility. Ginseng extract protected against spontaneous AR, indicating that it may stabilize membranes and may have influence on ion flux via interaction with P₄.

5. Conclusions

ER α and ER β exhibited much higher binding affinity for α -zearalenol and E₂ than for ginseng. Red ginseng refers to ginseng that has been steamed or sun-dried, which may alter the

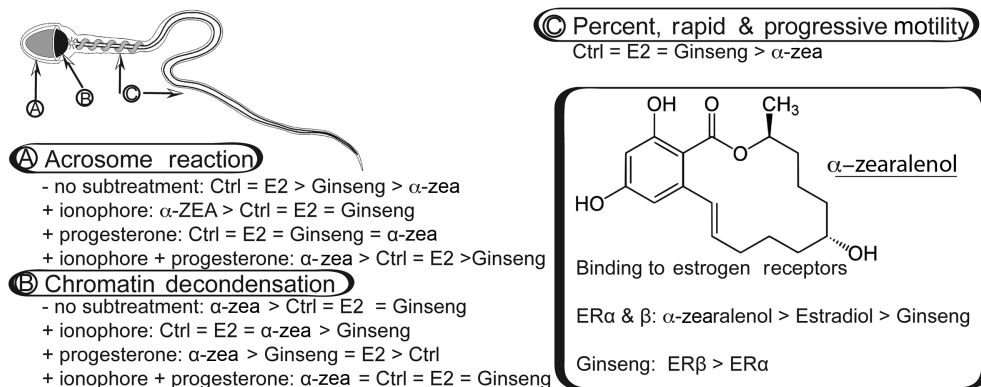


Fig. 3. Illustrated effects of ginseng, estradiol, and α -zearalenol on boar spermatozoa. Ctrl, control; α -Zea, α -zearalenol; E2, estradiol; ER, estrogen receptor.

ginsenoside content and result in a more potent product as evidenced by the increased binding to ER β . Gut microflora may also play an important role in the absorption and availability of ginseng. The adaptogenic activity of ginseng may result from its ability to crosstalk with numerous signaling pathways that act downstream of ER [106].

Ginseng-treated spermatozoa from boars of high fertility did not significantly differ from controls in the percentage of overall, progressive, and rapid motile cells, in kinematic parameters or in induction of AR in response to ionophore. Ginseng may have had a more noticeable effect on motility in spermatozoa with lower fertility by compensating for intrinsic damage [70,71]. Further experiments using knockout or silencing models may provide additional information on the involvement of classical ERs, splice variants, as well as nonclassical membrane pathways on sperm function and toxicology.

In conclusion, ginseng decreased chromatin decondensation in response to ionophore and decreased AR in response to P₄ and ionophore (see Fig. 3). α -Zearalenol decreased motility and kinematic parameters compared to controls, decreased spontaneous AR, and increased chromatin decondensation. These results indicate that ginseng and α -zearalenol influence multiple systems that regulate the reproductive fitness of spermatozoa.

Conflicts of interest

The authors declare that there is no conflict of interest.

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