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Research article

Effects of *Panax ginseng*, zearalenol, and estradiol on sperm function

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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 (ERα and ERβ)—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 (E_2)- 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 (P_4) and ionophore.

Conclusion: Zearalenol has adverse effects on sperm motility and function by targeting multiple signaling cascades, including P_4, E_2, 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 Ca^{2+}-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 (E_2) is found in the reproductive tract fluids of males and females. E_2 seems to be required for normal germ cell development; however, exceeding this level can cause dysfunction [6,7]. Nonfertile men had higher concentrations of E_2, with 53% having seminal estradiol concentrations above the 90th percentile value for fertile men [8]. Spermatozoa are exposed to varying levels of E_2 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 E_2 is difficult to accurately determine for an intracrine mechanism [9]. The seminal plasma of breeding boars had 92 pg/mL E_2 and the seminal plasma of bulls was found to contain 568 pg/mL [10]. Concentrations of E_2 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, E_2 concentrations of 1–10 μg/mL have been used in culture media [13–15].

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Concentrations of E_2 at 0.1 μg/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 ERα [32,33]. When two of the main ginsenosides (Rg1 and Rb1) did not account for the appreciable binding to ERα or ERβ, 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 ERα and ERβ, 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 1,800*g* for 15 min, and the supernatant removed. The pellet was resuspended in 8 mL solvent and mixed overnight. Supernatants were combined, placed in a ThermolyneDri-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 400*g* for 5 min to remove the extender. Spermatozoa samples were washed twice with 0.2mM $NaHCO_3$ 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 $2–3 \times 10^7$ 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 μg/mL; (2) ginseng extract, 2 mg/mL; and (3) α-zearalenol, 10 μg/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 1,800*g* 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 × 75 mm polypropylene tubes that contained test

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