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Impact of kudzu and puerarin on sperm function

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ABSTRACT

The goal of this study was to investigate the impact of kudzu (*Pueraria mirifica*) and the isoflavone puerarin in functional toxicological tests on spermatozoa and to assess the affinity of extracts and pure isoflavones for estrogen receptor (ER)-alpha and -beta (ERα, ERβ) in receptor binding assays. Capacitation, acrosome reaction and chromatin decondensation in spermatozoa were analyzed using microscopic analysis. Kudzu, but not puerarin, reduced motility of sperm. Puerarin reduced the percent spontaneous acrosome reaction in spermatozoa. The pathways used by kudzu that affect sperm function are not fully mirrored by puerarin. Puerarin, kudzu and its other phytoestrogenic components displayed preferential affinity for ERβ, however the diverse effects of kudzu and puerarin on sperm function implicate the involvement of multiple signaling systems.

1. Introduction

Despite the extensive history of medicinal plant use, it is estimated that only 5–15% of the approximate 250,000 species of higher plants have been chemically, pharmacologically and toxicologically investigated [1]. Similarities between chemical messengers in plant and animal signaling pathways suggest that inter-system activities are not to be unexpected. Kudzu is one of several plants that possess a group of compounds called phytoestrogens that exhibit hormone-like activity across biological systems by interacting with estrogen receptors (ER) [2,3]. Diverse and different physiological processes are regulated by classical and non-classical estrogen signaling pathways. The goal of this study was to investigate the toxicological impact of kudzu and puerarin on boar spermatozoa and determine if kudzu and puerarin displayed affinity for ER-alpha (ERα) and ER-beta (ERβ).

1.1. Kudzu

Kudzu is classified in the genus *Pueraria*, comprising 15 species in the Fabaceae family. The earliest written records on *Pueraria* document kudzu as a source of fiber, food, medicine, and fuel [4,5]. The root, stem, leaf, flower and seed of kudzu have also been used in traditional Chinese medicine [6]. The estrogenic isoflavones daidzein and daidzin were responsible for the anti-alcohol effect of kudzu in a Golden hamster animal model [7]. Kudzu extracts are available commercially in teas, soaps, and gums and are promoted for cosmetic enhancement, as well as cardiovascular and bone health [8–10]. To ascertain the mechanism of action for the hormone-like activity in kudzu and possible evidence of synergism between compounds, extracts and pure isoflavones were investigated in ERα and ERβ binding assays.

Phytoestrogens can act as endocrine disruptors in some situations and have been associated with adverse effects in the reproductive systems of animals. In women, high soy isoflavone supplementation can cause erratic elevations of plasma estradiol (E_2) throughout the menstrual cycle [11–13]. In rats, crude powdered and butanolic extracts of *P. tuberosa* had antifertility effects decreased testosterone activity and diminished production of competent spermatozoa [14]. The effects of kudzu extracts and an isoflavone component, puerarin, on competent spermatozoa from a domestic animal warranted further investigation.

1.2. Estrogens and phytoestrogens

Phytoestrogens are natural compounds capable of inducing biological responses in mammals that mimic or modulate the actions of endogenous estrogens, usually by binding to ER [15]. Although the chemical structures of phytoestrogens vary, most are structurally similar to the 18-carbon steroid structure of the estrogen backbone and possess comparable distances between hydroxyl groups essential for binding to ER [16,17]. Binding of a ligand is the first step in a chain of events that leads to a receptor-mediated response. Receptor-ligand binding assays are used to assess the

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relative binding affinities (RBA) of ligands, and parallels are drawn between the RBA of a compound and its biological potency.

The most bioactive form of estrogen in animals is E_2. Two distinct classical forms of the ERα and ERβ, have been identified in vertebrates [18]. There are differences in the two receptors related to structure, function, tissue expression and ligand binding, with many phytoestrogens having higher affinity for ERβ than ERα [2,19].

1.3. Spermatozoal signaling and toxicity

The spermatozoon is sensitive to environmental and toxicological insult. A complex interplay of signaling factors control spermatozoa motility and subsequent activities related to reproductive fitness. Changes in spermatozoa motility, membrane stability and chromatin decondensation are examined to determine a chemical's cellular toxicity. The mammalian spermatozoa can be used to study the effects of plant compounds on cellular and mitochondrial bioenergetics, membrane dynamics and chromatin stability.

1.3.1. Estrogens and spermatozoa

Developing germ cells and spermatozoa contain ERα and ERβ. Porcine and other mammalian spermatozoa contain the capacity to produce E_2 *via* p450 aromatase and the ability to respond to E_2 *via* ER [20,21]. Estrogen signaling pathways in spermatozoa may also involve a membrane estrogen receptor (MER) [22]. Nongemonic signaling by E_2 through a nongenomic ER induced a rapid increase of intracellular Ca^{2+} and caused a reduction in the progesterone (P_4) induced Ca^{2+} response [23]. Although E_2 had no direct effect on the acrosome reaction (AR), its interference with the P_4-mediated Ca^{2+} flux ultimately resulted in inhibition of AR.

In somatic cells, ER signaling pathways crosstalk with PKA, PKC and phosphatidylinositol-3-OHKinase/Akt/PKB pathways [24–26]. The presence of these systems within spermatozoa suggests that phytoestrogen compounds in kudzu may directly or indirectly affect spermatozoa function by modulating cAMP (PKA), Ca^{2+} (PKC) or general kinase activity [22,27,28].

1.3.2. Sperm motility

Spermatozoa motility is dependent on cAMP and Ca^{2+}-dependent signal transduction pathways modulating mitochondria and cellular function [29,30]. Analysis of sperm motility and sperm-head motion may reflect changes in cellular and flagellar function and membrane integrity of the spermatozoa. The computer assisted sperm analyzer (CASA) analyses spermatozoa movement parameters [31–35]. Comparisons of subjective semen analysis with CASA suggest the automated method is a rapid, objective, and reliable alternative to conventional semen analysis [33,36–39].

The relationship of CASA derived parameters with spermatozoal function has been documented; mitochondrial potential in spermatozoa is positively correlated with increases in motility, average path velocity (VAP), curvilinear velocity (VCL), and percent hyperactive [40]. Human fertility potential *in vitro* was correlated with sperm motility parameters measured with CASA, including amplitude of lateral head displacement (ALH), VCL, linearity (LIN), straight line velocity (VSL) and rapid motility [36,41–46]. In addition to VCL and VAP, sperm hyperactivation has been shown to be an important marker of fertilizing ability [41,46]. Spermatozoa velocity parameters and motion parameters have also been used to determine the effect of toxins on sperm function [47].

1.3.3. Capacitation and acrosome reaction (AR)

The head region of a spermatozoon contains the nucleus with densely packed inactive DNA complexed with highly basic proteins known as protamines and the acrosomal cap, a membrane-bound vesicle containing a matrix of hydrolytic enzymes. Under specific physiological conditions, the spermatozoon undergoes the AR, an exocytotic event in which the acrosomal cap membrane releases hydrolytic enzymes that facilitate spermatozoa penetration of the oocyte [48,49].

Prior to becoming fertilization-competent, mammalian spermatozoa undergo a series of physiological changes known as capacitation [50–52]. Capacitation of spermatozoa is associated with increased cellular $[Ca^{2+}]$ and cAMP production and membrane destabilization.

In vivo, the AR occurs when the spermatozoa come in contact with the zona pellucida of the oocyte. The AR can also be induced *in vitro* in capacitated spermatozoa by incubation with solubilized zona pellucida or with ligands such as P_4, epidermal growth factor, and atrial natriuretic peptide [53–56]. The first step in the AR, after the spermatozoa are capacitated, is an influx of exogenous Ca^{2+} ions across the sperm membranes. The use of a Ca^{2+} transporting agent, such as, ionophore A23187, results in stimulation of the AR [57,58]. Capacitation and AR were stimulated in uncapacitated cells by E_2, and the xenoestrogens genistein, 8-prenylnaringenin and nonylphenol [59]. In capacitated cells, E_2 had no effect, but the other three compounds significantly increased AR rates.

1.3.4. Chromatin decondensation

During mammalian spermiogenesis, spermatozoa chromatin undergoes condensation characterized by replacement of histones with protamines [60]. The DNA-protamine complex enables genetic material to become highly condensed and resistant to mechanical and chemical damage. Normally, sperm chromatin decondensation occurs after penetration of the oocyte when protamine is replaced by embryonic histones [47,61–64]. Nuclear chromatin decondensation of spermatozoa is essential for fertilization and normal embryonic development.

Premature decondensation in spermatozoa, that is, unwinding of the chromatin before fertilization, may be an indication of DNA or chromatin damage. Spermatozoa with unstable chromatin are associated with decreased fertility possibly due to decreased resilience and/or abnormal rates of decondensation [65].

2. Methodology

2.1. Plant material and extraction

Kudzu (*P. mirifica*) was a generous gift from Dr. J. L. McLaughlin (Nature's Sunshine Products, Inc.; Spanish Fork, Utah). 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 with occasional shaking, then filtering through Whatman # 1 filter paper. The extraction was repeated again on the pellet and filtrates combined. The filtrate was evaporated to approximately 1–3 ml under steady stream of N_2, transferred to a 5-ml bottle and evaporated to dryness. The dried extract was weighed (W_2), the percentage of extract from sample calculated ($W_1/W_2 \times 100$), and shipped to the Endocrine Physiology Laboratory at Clemson University; Clemson, S. C. for analysis in the receptor-binding and cell-based assays.

Dried extracts from Nature's Sunshine equivalent to 2 g original ground plant were suspended overnight in 3 ml hexane. Then, 3 ml of 80% methanol were added to the hexane plant suspension and the mixture shaken for 1 h. Samples were centrifuged at $1800 \times g$ 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)) and evaporated to dryness on a heated DriBath (45 °C) under a gentle stream of filtered