Exposure to genistein does not adversely affect the reproductive system in
adult male mice adapted to a soy-based commercial diet

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Genistein, a soybean-originated isoflavone, is widely consumed by humans for putative beneficial health effects but its estrogenic activity may affect adversely the development of male reproductive system. Five-week-old ICR mice were purchased and fed with a soybean-based Purina Chow diet until 6 months of age. The animals were exposed by gavage to genistein (2.5 mg/kg/day) or 17β-estradiol (7.5 µg/kg/day) for five weeks. Corn oil was used for the negative control. The animals were fed the casein-based AIN-76A diet throughout the experimental periods. There were no significant differences in body and organ weights of mice among experimental groups. No significant differences in sperm counts and sperm motile characteristics were found between the control and the genistein groups. Treatment of 17β-estradiol caused a significant decrease in epididymal sperm counts compared to the control (p < 0.05). The level of phospholipid hydroxide glutathione peroxidase in the epididymis of mice exposed to genistein was significantly higher than that of the control mice (p < 0.05). 17β-estradiol treatment caused a reduction of germ cells in the testis and hyperplasia of mucosal fold region in the prostate of mice. Genistein treatment did not cause any lesion in the testis, epididymis, and prostate. These results suggest that dietary uptake of genistein at adult stage of life may not affect male reproductive system and functions.

Key words: Estradiol, Genistein, Phospholipid hydroxide glutathione peroxidase, Sperm

Introduction

Genistein (4',5,7-trihydroxy-isoflavone), the principal soy isoflavone, has been the subject of numerous studies in experimental animals and humans because of possible beneficial and adverse health effects due to estrogenic activity [25]. Epidemiological studies have revealed that individuals who consume a traditional diet high in soy products have a low incidence of certain types of cancer, such as breast, prostate and colon cancer [1]. Diets high in soy contain multiple agents that may contribute to the effect. Nonetheless, much research attention has focused on the isoflavones and particularly genistein, as active compounds responsible for the beneficial effects of soy [4]. In the typical Asian diet, 1.5 mg/kg/day of genistein or other isoflavones may be ingested, whereas the typical Western diet contains less than 0.2 mg/kg/day [6]. The health benefits of soy isoflavones may be due to the presence of estrogenicity and/or anti-estrogenicity and other biological activities such as inhibition of angiogenesis, cell proliferation, tyrosine kinase activity, free radical production, and steroid metabolizing enzymes [2,15,32].

Research assessing the potential adverse effects associated with isoflavone consumption is primarily directed toward defining any potential risk from exposure to a range of doses of isoflavones during different life stages. There has been considerable debate over the possible risk and/or benefits of isoflavone consumption during the sensitive stages of fetal and infant development, because of the weak estrogenic activity of genistein and other isoflavones [18,29]. Strauss et al. [27] reported that in adult male mice, genistein induced the typical estrogenic effects in doses comparable to those present in soy-based diets, while in neonatal animals, considerably higher doses are required to show estrogen-like activity. These findings have raised concern over exposure of human to significant doses of soy isoflavones at various
stages of development.

There are many recent research papers on effects of early exposure to genistein on the reproductive functions [9,11,17,21]. Several papers showed no adverse effects of genistein on animal reproductive systems at the human intake dose level [11,12,17,19]. Meanwhile, some showed adverse effects of genistein on reproductive function after puberty in animals [9,21,33]. The discrepancy in these results may be due to differences in time, duration, and dose of exposure to genistein and/or use of animal species and strains. Meanwhile, data for the exposure to genistein at adult stage is very limited.

The objective of the present study was to evaluate whether genistein causes adverse effects on reproductive system as exposed for 5 weeks at adult stage of mice adapted to a soy-based Purina Chow diet until 6 months of age. The animals were fed with a casein-based AIN 76A diet during the experimental period of 5 weeks. Changes in the weight and histopathology of reproductive organs, sperm count and sperm motility, and levels of phospholipid hydroxide glutathione peroxidase (PHGPx) mRNA expression were investigated.

Materials and Methods

Chemicals

Genistein (purity, >98%), 17β-estradiol, and corn oil were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Genistein was diluted with corn oil and mixed vigorously prior to use. The other chemicals and reagents used in this study were also purchased from Sigma and were of the highest grade commercially available.

Laboratory animals

Five-week-old ICR mice were purchased from Daehan Inc. (Seoul, Korea) and housed in polycarbonate cages with wood chip bedding for about 5 months until use of experiment (6 months old). The animal facilities were maintained under controlled conditions with temperature of 21 ± 2°C, relative humidity of 50 ± 10% and artificial illumination of a 12-hr light-dark cycle. All animals received humane care as outline with “Guide for the care and use of animals” (Chungbuk National University Animal Care Committee according to NIH #86-23). Animals were fed with a soy-based Purina Chow diet (Purina Korea, Seoul, Korea) until starting experiment. Six-month-old male mice were randomly divided into 3 experimental groups (10 mice per group) including corn oil (control), genistein (2.5 mg/kg), and 17β-estradiol (7.5 µg/kg). The animals were orally administered everyday with the test compounds for 5 weeks under the casein-based AIN-76A diet (Harlan Teklad, Madison, WI, USA). Animals were sacrificed under anesthesia with ethyl ether, and their reproductive organs including testis, epididymis and prostate were removed and weighed.

Sperm counts in testis and cauda epididymis

Testicular parenchyma tissue was displaced in 12 ml distilled water at 4-6°C. The tissue was homogenized at a low speed for 1.5-2 min using a polytron homogenizer (Omni 5000 International Co, Waterburg, CT, USA) and sonicated for 3 min at 4°C. Cauda epididymis was chopped with a sharp scissor and homogenized with a low speed in 10 ml distilled water for 1.5-2 min at 4-6°C. The number of homogenization-resistant spermatids was enumerated using a hemocytometer.

Analysis of sperm kinematics

The working medium for mouse sperm kinematics was a modified Tyrode’s solution [31], as described by Holloway et al. [16]. It was equilibrated overnight to a pH of 7.35 ± 0.5 in a 5% CO2 incubator at 37°C. For sperm motility assessment, the medium was modified with the addition of 0.4% bovine serum albumin (BSA) and equilibrated to a pH of 7.35 ± 0.5. Each testis and ex-current duct was immediately recovered by a midline incision. Caudal epididymis and vas deferens were dispersed, dissected free of the epididymis and surrounding fat, and washed in media. The epididymis was placed in 3 ml of the modified Tyrodes medium supplemented with 0.4% BSA in a 35 mm plastic petri dish at 37°C. After the tissue was removed, sperm suspension was collected, gently mixed, and kept at 37°C in a 5% CO2 atmosphere. Aliquots of the sperm suspension were diluted with fresh medium to adequate concentration. The aliquots of 30 ml were placed in pre-warmed slide chambers with the depth of 20 mm. The slide chambers were transferred to heated plate of an inverted phase-contrast microscope (Olympus IX 70, Tokyo, Japan). PH2 condenser and 4X PH1 object lens were used to produce pseudo-dark-field views. Computer-assisted sperm motility analysis (CASA) was performed using a sperm image analysis system (SIAS, Medical supply Co. Seoul, Korea). The real-time of continuous image processing and data acquisition over extended periods was recorded. For each slide, the tracks of sperm in 10 fields were recorded for approximately 2-3 min [35]. Centroids were used for estimation of motion endpoint, which includes motility (number of sperm exceeding threshold minimum velocity/total number of sperm), curvilinear velocity (VCL: mean frame-to-frame velocity), straight-line velocity (VSL: velocity between centroids in first and last frame tracked), average path velocity (VAP: velocity obtaining from smoothing the original path), hyper-activated sperm (HYP), beat cross frequency (BCF: frequency that centroid crosses average trajectory), mean angular displacement (MAD: time-average of absolute values of the instantaneous turning angle of the sperm head along its curvilinear trajectory), lateral head displacement (ALH: displacement of the centroid from a computer-calculated average trajectory). Linearity (LIN: [VSL/VCL] × 100), straightness (STR: [VSL/VAP] × 100), and dance (DNC: VCL × ALH) were calculated with above
parameters. These parameters have been modeled and refined mathematically to describe the motion of each spermatozoon as it travels through a microscopic dark field [3].

Histopathological evaluation

Body weights (every week) and sex organ weights including testis, epididymis, and prostate were measured. Testis, epididymis, and prostate were fixed in Bouin’s fixative and washed with saturated lithium carbonate in 70% ethyl alcohol to remove excess of the fixative. After normal tissue processing using an automatic tissue processor (Shandon Hypercenter XP, Houston, TX, USA) and an embedding center (Leica, Solms, Germany), the organ tissues were stained with hematoxylin and eosin (H & E) and examined microscopically.

Total RNA isolation and RT-PCR

Total RNA was extracted from testis, epididymis, and prostate using the TRIzol reagent (Life Technologies, Gaithersburg, MD, USA), according to the manufacturer’s instruction [22]. The RNA pellet obtained in the final step was dissolved in 50 ml of sterile diethylpyrocarbonate (DEPC)-treated water and its concentration was determined by a UV spectrophotometer at 260 nm. RNA was kept in DEPC-treated water at −70°C until use. Reverse transcription of mRNA and amplification of cDNA were performed using a Pelter thermal cycler (MJ Research Inc., Waltham, MA, USA). Total RNA (1.0 mg) was synthesized by using the 1st strand cDNA synthesis kit (Boehringer Mannheim, Germany) following the manufacturer’s instruction. The PCR mixture was made as the following: 0.15 ml of TaqGold DNA polymerase (Perkin Elmer; Boston, MA, USA), 1.0 ml of sense primer (5’-ATGCA CGAAT TCTCA GCCAA G-3), 1 ml of antisense primer (5’-GGCCG GTCCT TCTCT AT-3), 2.5 ml of dNTPs, 2.5 ml of 10-strength PCR buffer containing 1.5 mM MgCl2, and 1 ml of template cDNA in 16.85 ml of ultra-distilled water. PCR amplification was carried out in the thermal cycler using a protocol of initial denaturing step at 95°C for 10 min; then 35 cycles at 95°C for 1 min (denaturing), at 55°C for 1 min (annearing), and at 72°C for 1.5 min (extension); and a further extention at 72°C for 10 min. The PCR products were run on a 2% agarose gel in Tris- borate-EDTA buffer. Every sample also tested for RNA integrity by using GAPDH primers: sense primer (5’-AACGG ATTTG GTGGT ATGGG-3), antisense primer (5’-AGCCT TCTCC ATGT GGTGA AGAC-3). Expected PCR products sizes of PHGPx and GAPDH were 462 and 302 bp, respectively. The relative absorbance of specific mRNA was normalized to the relative absorbance of GAPDH mRNA.

Statistical analysis

Data were analyzed using SAS program for ANOVA. The significance of difference between the mean of each treatment group and that of control group was evaluated statistically by least significant difference (LSD) at the level of \( p < 0.05 \) and \( p < 0.01 \).

Results

Body and organ weights

Changes in body weights are shown in Fig. 1. There was no significant difference in body weight among experimental groups (Fig. 1). Relative organ weights of testis, epididymis, and prostate in mice exposed to genistein were not significantly different from the control (Fig. 2).
Sperm count and sperm motility
Exposure to genistein for 5 weeks at adult stage did not affect sperm counts in the testis and epididymis (Fig. 3). 17β-estradiol treatment caused a significant decrease in sperm counts in the epididymis by about 42% compared to the control ($p < 0.05$). Testicular sperm count was also decreased by the treatment of 17β-estradiol but it was not significantly different from the control (Fig. 3). Sperm motile characteristics including MOT, VCL, VSL, VAP, HYP, BCF, MAD, and ALH were not changed by genistein exposure (Fig. 4). Meanwhile, 17β-estradiol treatment slightly decreased all the sperm motile characteristics (Fig. 4).

PHGPx mRNA expression
As shown in Fig. 5, exposure to genistein at adult stage significantly increased PHGPx mRNA expression in the epididymis, compared to the control ($p < 0.05$). The PHGPx expression in the epididymis was much higher by genistein than by 17β-estradiol (Fig. 5). There were no significant differences in the expression of PHGPx mRNA in testis and prostate among experimental groups (Fig. 5).

Histopathological findings
17β-estradiol treatment caused remarkably the presence of detached germ cells in seminiferous tubules and reduction of germ cells in the testis (Fig. 6C). 17β-estradiol also caused cytoplasmic vacuolization of sertoli cells (Fig. 6C). Exposure to genistein did not cause any change in the testis, epididymis, and prostate (Fig. 6A, 7A, & 8A). The 17β-estradiol treatment also caused the hyperplasia of epithelial cells and proliferation of interstitial connective tissue in the prostate (Fig. 8C).

Discussion
An early exposure to exogenous estrogenic chemicals can disrupt male reproductive development and impair fertility at later stages of life [5,7,26]. Many rodent diets contain compounds such as soy isoflavones known to have estrogenic properties [8]. The dietary background of phytoestrogens may modulate some responses to environmental estrogens when these compounds are tested in rodent bioassay [28]. In the present study, exposure to genistein at adult stage of mice adapted to a soybean-based diet was carried out daily by oral gavage for 5 weeks and the animals were fed with a casein-based open formula (AIN-76A) purified diet with non-detectable levels of estrogenic isoflavones throughout the experiment [30]. Our study clearly showed that exposure to genistein at adult stage of mice did not affect male reproductive functions including sperm counts and sperm quality. The exposure to genistein did not cause any change in relative weights and
The effects of genistein on reproductive development in animals are still controversial. Several reports showed that maternal exposure to genistein at the reliable dose of human intake during gestation and/or lactation has no adverse effects on live pups number, implantation sites number, sex ratio, anogenital distance, eyelid/vaginal opening, and body weight of live pups as well as reproductive organs weight and gametogenic function in F1 male offspring [11,17,24]. In addition, neonatal exposure to genistein at 40 mg/kg/day during birth and lactation did not affected development of male reproductive organs [12,19].

Meanwhile, adverse effects of genistein on reproductive system have been also reported [9,21,33]. Oral exposure to genistein during puberty decreased body weights of offspring [21]. Dietary exposure of genistein to pregnant and lactating dams starting on gestation day 7 also affected function and histology of reproductive organs in both female and male pups [9]. Wisniewski et al. [33] also reported that perinatal exposure to genistein resulted in transient and lasting alterations in masculinization of the reproductive system in male rats. These adverse effects may be due to ability of genistein to cross placenta and to reach fetal brain from maternal serum genistein levels that are relevant to those observed in humans [10]. These reports suggested that dietary genistein ranges available in humans produced effects in multiple estrogen-sensitive tissues in males and females that are generally consistent with its estrogenic activity [9].

Strauss et al. [27] reported that in adult male mice, genistein induced the typical estrogenic effects in doses comparable to those present in soy-based diets, while in neonatal animals, considerably higher doses are required to

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**Fig. 5.** PHGPx mRNA expression patterns in male adult mice exposed to genistein and 17β-estradiol for 5 weeks. Genistein 2.5 mg/kg (1), 17β-estradiol 7.5 µg/kg (2), Control (3). cDNAs of testis, epididymis and prostate loaded 2.0 % agarose gel. A) A representative expression of PHGPx mRNA and B) the corresponding GAPDH mRNA. The ratios of PHGPx and GAPDH bands were calculated. Values represent mean ± SD (n = 10). *p < 0.05; compared to the control.

**Fig. 6.** Histopathology of testis in male adult mice exposed to genistein and 17β-estradiol for 5 weeks. (A): Control, (B): Genistein (2.5 mg/kg/day), (C): 17β-estradiol (7.5 mg/kg/day). Detachment of germ cells from epithelium and cytoplasmic vacuolization of sertoli cells. H & E, x100.
show estrogen-like activity. Our results showed that the exposure to genistein at 2.5 mg/kg/day for 5 weeks had no adverse effects on reproductive system at adult stage of mice. However, 17β-estradiol treatment induced severe impairment in male reproductive system even at the adult stage of mice. Although the exposure to genistein induced no changes in the testis, epididymis, and prostate of mice, the estrogenic activity of genistein may not be excluded. Our study also showed that genistein exposure significantly increased PHGPx expression in the epididymis, probably due to protection against or compensation for damage by the estrogen-like compound. Nam et al. [22] reported that 17β-estradiol increased PHGPx expression in the testis and prostate of rats, suggesting that estrogen might regulate PHGPx transcription in male reproductive organs.

Sperm motility is an important factor to maintain fertilization. Genistein inhibits the induction of acrosomal exocytosis and binding of spermatozoa to the zona pellucida (ZP) [34]. ZP-induced acrosomal exocytosis in domestic cat
sperms are regulated via a tyrosine kinase-dependent pathway, suggesting that a defect in the signaling pathway may cause a compromised sperm dysfunction [23]. Genistein, an inhibitor of protein phosphorylation and dephosphorylation, may play regulatory roles in mediating sperm capacitation [13]. A previous in vivo report showed that genistein inhibits tyrosine phosphorylation of sperm tail protein and blocks capacitation and subsequently sperm hyperactivity [20]. The in vivo effect may be associated with a decrease in fertility ability of sperm. However, many reports have showed that genistein has no effects on sperm motility parameters [14,17,21]. In the present study, the exposure to genistein slightly increased sperm motile characteristics compared to the control. Fielden et al. [11] reported that the exposure to genistein at the dose of 10 mg/kg/day significantly increased in vitro fertilizing ability of epididymal sperm by 17% [11]. Although several reports indicate adverse effects of genistein on the reproductive system, our results suggest that daily intake of genistein has no observable detrimental effects on male reproductive system. The present study extends our knowledge of the effects of genistein exposure at adult stage on male reproductive system and may have implications for human health in terms of potential relationships of endocrine disrupters and urogenital abnormalities thought to be increasing in incidence in men.

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