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Uterine phenotype of young adult rats exposed to dietary soy or genistein during development☆

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Abstract

Dietary soy intake is associated with protection from breast cancer, but questions persist on the potential risks of the major soy isoflavone genistein (GEN) on female reproductive health. Here, we evaluated intermediate markers of cancer risk in uteri of cycling, young adult Sprague–Dawley rats lifetime exposed to one of three AIN-93G semipurified diets: casein (CAS), soy protein isolate (SPI^+ with 276 mg GEN aglycone equivalents/kg) and CAS+GEN (GEN at 250 mg/kg). Postnatal day 50 (PND50) rats lifetime exposed to GEN or SPI^+ had similar uterine luminal epithelium height, myometrial thickness, endometrial gland numbers, endometrial immunoreactive proliferating cell nuclear antigen (PCNA), and serum estrogen and progesterone, as CAS-fed rats. GEN-fed rats showed modestly increased apoptosis in uterine glandular epithelium, compared to those of CAS- or SPI^+-fed groups. Diet had no effect on the uterine expression of genes for the tumor suppressors PTEN, p53 and p21, and the apoptotic-associated proteins Bcl2, Bax and progesterone receptor. Uterine tissue and serum concentrations of total GEN were higher in rats fed GEN than in those fed SPI^+. Human Ishikawa endocarcinoma cells treated with GEN-fed rat serum tended to exhibit increased apoptotic status than those treated with CAS-fed rat serum. Exogenously added GEN (0.2 and 2 μM) increased, while estradiol-17β (0.1 μM) decreased Ishikawa cell apoptosis, relative to untreated cells. Results suggest that lifetime dietary exposure to soy foods does not alter uterine cell phenotype in young adult rats, while GEN, by enhancing uterine endometrial glandular apoptosis in vivo and in vitro, may confer protection against uterine carcinoma. Given its limited influence on uterine phenotype of young adult females, GEN, when taken as part of soy foods or as supplement, should be favorably considered for other potential health benefits.

Keywords: Uterus; Soy; Genistein; Nutrition; Apoptosis; Endocarcinoma

1. Introduction

Numerous epidemiological studies have pointed to the breast cancer-protective effects of soy-based diets [1–4]. Although the molecular mechanisms underlying these effects remain ill-defined, this protection has been attributed, in part, to the soy isoflavone genistein (GEN) which exhibits estrogenic agonist/antagonist activity through estrogen receptor (ER)-α- and ER-β-mediated pathways [5,6]. The uterus, an estrogen-sensitive tissue, is also considered a target of GEN action [7,8]. However, a consensus on the positive or negative influences of GEN on uterine function remains lacking as studies to date using animal models have differed on the dose, age of first exposure, and duration and route of exposure for assessing possible benefits or risks [9–15]. Thus, while exposure to GEN has been shown to negatively impact the development of the female genital tract and subsequent fertility in a number of studies [9–12], other studies, by contrast, have demonstrated the lack of adverse reproductive effects of GEN, when taken in the diet or by injection [13–15]. Further, while subcutaneous administration of GEN to neonatal mice was associated with increased incidence of uterine adenocarcinoma [16], GEN administered by the same route was

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found in another study to be protective against estrogen-induced endometrial carcinogenesis in mice [17].

There have been no reports in humans on the adverse health effects from exposure to isoflavones in utero and postnatal. Asians, who are regular consumers of soy foods, have a lifetime of exposure to soy phytochemicals such as GEN; this level of exposure is relatively high in utero due to placental transfer of these phytochemicals from maternal circulation, decreases to very low levels during infancy as phytochemicals do not cross into the breast milk and significantly increases postweaning after initiation of traditional diets rich in soya products [18]. Female offspring of Asian women who consume GEN as part of a normal soya diet have been suggested to benefit from habitual soy intake [2–4,19–21], although limited direct evidence exists to link this to protection from risk of uterine dysfunctions such as endometrial carcinoma and endometriosis [22]. Indeed, only one epidemiological study has reported an analytical association between ingestion of phytoestrogenic compounds such as GEN and reduced risk of endometrial cancer [19]. Nevertheless, the potential health benefits of soy have encouraged women in the Western world to supplement their regular diets with GEN as a substitute for regular soy food consumption. However, the popular association of GEN and soyfoods is not entirely accurate; while native soy proteins have predominantly the isoflavone conjugate genistin, which undergoes intestinal metabolism to the aglycone and other molecular forms upon ingestion, GEN available in health food stores exists solely as the aglycone component. Moreover, soy proteins are also a rich source of the isoflavone daidzen, which has been demonstrated to exhibit biological effects in animal models in vivo and carcinoma cells in vitro [17,23,24].

In the United States, where newborns consuming commercially available soy formula are exposed to high (micromolar) levels of GEN for a limited period in the first year of life [25,26], the adverse consequence of this early GEN exposure on long-term uterine health remains a major concern. Thus, information gleaned from an analysis of molecular and cellular parameters modified in the young adult uterus by prolonged and early exposure to dietary soy proteins and associated isoflavones, a regimen simulating the Asian diet may aid in resolving the issues surrounding their effects on women's reproductive health. In the current study, we evaluated intermediate markers of cancer risk, namely, proliferation status, apoptotic index and expression of specific growth regulatory genes, in uteri of cycling, young adult Sprague–Dawley rats lifetime exposed to a diet of soy protein isolate (SPI^+) or a control diet (CAS) supplemented with GEN, relative to those fed the control CAS diet. Further, we compared the apoptosis-inducing effects of exogenously added GEN, estradiol-17β and of sera from rats fed GEN-enriched diets using human Ishikawa epithelial carcinoma cells to discern the potential of GEN for cancer chemoprevention in the endometrium, as has been suggested for the mammary gland [1,18,27].

2. Materials and methods

2.1. Rats, diets and tissue collection

All animal procedures were approved by the University of Arkansas for Medical Sciences Animal Care and Use Committee. Time-mated Sprague–Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) and kept individually in polycarbonate cages in rooms under controlled temperature (24°C), humidity (40%) and light (12-h light/dark cycle). Rats at gestation day (GD) 4 were randomly assigned to semipurified isocaloric diets which were made according to the AIN-93G diet formula [28], except that corn oil replaced soy bean oil and contained as sole protein source, either casein [20% (w/w) CAS; New Zealand Milk Products, Santa Rosa, CA] (Group 1) or soy protein isolate [20% (w/w) SPI+] (a gift from Solae Company, St. Louis, MO) (Group 2). Diet containing SPI^+ had 276 mg GEN (aglycone) equivalents per kilogram. A third diet group (Group 3) contained CAS [20% (w/w)] as sole protein source to which was added aglycone GEN (Sigma, St. Louis, MO) at 250 mg/kg; the latter resulted in serum GEN levels (~1.5 μM; Table 1) that were within the range of those found in humans consuming a normal soya diet [29]. Animals were provided food and water ad libitum. At delivery, pups were randomly selected from litters of five to seven dams of the same diet group, and 10 pups (five males and five females) were assigned to each dam for suckling. The offspring were weaned to the same diets as their mothers and were continued on these diets throughout the study. We have previously reported that females fed these diets exhibited normal 4- to 5-day estrous cycles, as evaluated by daily vaginal smears beginning at postnatal day (PND) 32 [18,30,31]. Moreover, females in all three groups consumed the same amounts of food, and their body weights did not significantly differ with diet from birth until PND50 (data not shown). At PND50, females ($n=10$ per diet group) were sacrificed, and uteri were collected and weighed. For each female, the left uterine horn was immediately homogenized in TriZol reagent (Invitrogen, Carlsbad, CA), and the homogenate frozen at −80°C for later RNA isolation, while the right horn was fixed overnight in 10% neutral buffered formalin for subsequent morphometry and immunohistochemistry. The male rats were used in an unrelated study.

Table 1
Total genistein content in PND50 rat uterus and serum as a function of diet[a]

Diet	Tissue[b] (nmol/g)	Serum[c] (μmol/L)
CAS	0.02±0.01	0.01±0.01
SPI^+	0.33±0.11*	0.44±0.10*
CAS+GEN	0.63±0.24*	1.47±0.04*

[a] Values are mean±S.E.M.
[b] Tissues were from $n=4$ individual rats per diet group.
[c] Sera were pooled from $n=6$ rats per diet group and were analyzed in triplicate.
* Different from CAS, $P<.05$ (t test).

2.2. Serum estrogen and progesterone levels

Trunk blood was collected into sterile tubes and centrifuged to obtain serum, which was stored at −20°C prior to analysis. Radioimmunoassays for estrogen (E) and progesterone (P) were performed using Coat-A-Count Estradiol or Coat-A-Count Progesterone diagnostic kits following the manufacturer's instructions (Diagnostic Products, Los Angeles, CA). All samples were analyzed in duplicate in the same assay.

2.3. Uterine morphometry, immunohistochemistry and TUNEL

Formalin-fixed uteri were embedded in paraffin, and sections (5 μm thick) were mounted on poly-lysine-coated microscope slides (Fisher Scientific, Pittsburgh, PA), deparaffinized and dehydrated in a graded alcohol series, followed by xylene. Sections were stained with hematoxylin and eosin following standard procedures. Uterine luminal epithelial height and myometrial thickness were measured using an MCID video microscopy system equipped with MCID+ software (Imaging Research, Ontario, Canada). Measurements were taken from five different areas per section, and two independent sections from each of four animals per diet group were analyzed. The number of uterine glands was counted in a visual field of ×200 magnification, and two independent sections from each of four animals per diet group were analyzed. Immunoreactive PCNA was detected after antigen retrieval, following previously described procedures from this group [32], using a mouse monoclonal antibody raised against rat PCNA (clone PC10, Dako, Carpinteria, CA; 1:600 dilution). Four randomly chosen fields (×200 magnification) per slide per rat, with two independent sections from each of four rats per diet group, were analyzed. An average of 1000 stromal cells, 300 luminal epithelial cells and 50 glands were counted for each uterine section, and only those cells showing a brown color were scored as positive for PCNA expression. To identify apoptotic cells, the terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick-end labeling (TUNEL) assay was performed on paraffin-embedded sections (4 μm thickness) (Oncogene, La Jolla, CA). TUNEL-positive cells were counted from three randomly selected fields (×200 magnification) per slide, and two slides were used for each rat. Tissue sections from six to seven animals per diet group were analyzed.

2.4. RNA isolation and quantitative real-time RT-PCR (QPCR)

Total RNA was extracted by homogenization of tissues in TriZol reagent. Integrity of the isolated RNAs was confirmed using the RNA6000 Nano LabChip kit with the Agilent 2100 Bioanalyzer System (Agilent Biotechnologies, Palo Alto, CA). The procedures for cDNA synthesis using a two-step RT-PCR reaction kit (Applied Biosystems, Foster City, CA), the design of PCR primers (for phosphatase and tensin homolog deleted in chromosome 10, PTEN; progesterone receptor, PR; $p21^{WAF}$; 18S rRNA) using PrimerExpress software (Applied Biosystems) and the conduct of QPCR using the SYBR Green detection system and an ABI Prism 7000 Sequence Detector (Applied Biosystems) followed previously described procedures from this group [32,33]. The rat PCR primers for p53, Bcl2 and Bax were, respectively, p53 [forward (F): 5′-AGCTGAATGAGGCCTTGGAA-3′; reverse (R): 5′-TGAGTCAGGCCCCACTTTCTT-3′], Bcl2 (F: 5′-GGAGGATTGTGGCCTTCTTTG-3′; R: 5′-GCCGGTTCAGGTACTCAGTCAT-3′) and Bax (F: 5′-AGGCGAATTGGCGATGAAC-3′; R: 5′-GCTGCCACACGGAAGAAGAC-3′). The relative transcript levels were calculated at cycle threshold values (Cτ) at which each signal was first detected above background, using the ABI Prism 7000 SDS Software (version 1.0) (Applied Biosystems). Standard curves were generated using serial dilutions of either pooled uterine cDNAs (for p53, Bax, Bcl2 and p21) or purified plasmid DNA containing the corresponding target gene as cloned cDNA (PR, PTEN). Melting curves were analyzed to determine the specificity of the amplified product and to confirm the absence of primer–dimer formation. Messenger RNA abundance was normalized to the corresponding 18S rRNA.

2.5. Cell culture and TUNEL

The human endometrial epithelial carcinoma cell line Ishikawa (courtesy of Dr. Bruce Lessey, Greenville, SC) was grown at 37°C in an atmosphere of 5% CO_2/95% air in minimal essential medium (MEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution (GIBCO, Carlsbad, CA). For TUNEL assay, Ishikawa cells at 1×10^4 density were seeded on immunofluorescence chamber slides (Fisher Scientific) in MEM containing 10% FBS. The following day, cells were washed with PBS and incubated overnight in MEM containing low serum (0.5% FBS). Treatments were added in fresh MEM/0.5% FBS. Twenty-four hours later, cells were rinsed three times with PBS, fixed in 4% formaldehyde for 1 h and then incubated with proteinase K (20 μg/ml final concentration in 10 mM TRIS, pH 8) for 5 min at room temperature to increase nuclear permeability. Incubation with fluorescein-labeled TdT reagent (Oncogene) was carried out in a humidified chamber at 37°C for 1 h, following the manufacturer's instructions. Labeled nuclei, representing apoptotic cells, were counted in three separate fields (×200 magnification) containing 200–300 cells each and were photographed with an Olympus IX-71 fluorescence microscope using a standard fluorescein filter.

Equal volumes of sera from PND50 female rats fed CAS or CAS+GEN diets ($n=6$ individual rats per diet) were pooled, filter-sterilized (70 μm acrodisc) and added to cells at a final concentration of 10% in MEM containing charcoal-stripped 0.5% FBS. Treatment of cells with GEN (0.2 or 2 μM) or estradiol-17β (0.1 μM; Sigma) was carried

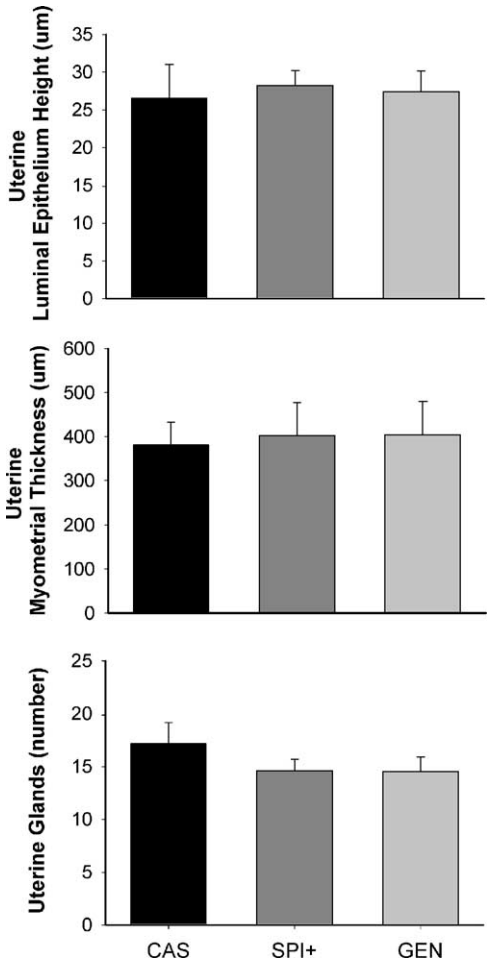

Fig. 1. Effect of diet on uterine growth parameters. Uterine luminal epithelium height (top panel) and myometrial thickness (middle panel) were measured in hematoxylin-and-eosin-stained uterine sections using an MCID video microscopy system equipped with MCID+ software. Measurements (mean±S.E.M.) were taken from five different areas per section and two independent sections from $n=4$ PND50 rats per diet group. Endometrial gland numbers (bottom panel) were counted in randomly chosen visual fields of ×200 magnification. Data (mean±S.E.M.) were obtained from analysis of uterine sections (two fields per section) from $n=4$ PND50 rats per diet group. No differences were observed in these parameters among the diet groups.

out in MEM containing charcoal-stripped FBS (0.5%). The apoptosis assays were performed in two independent experiments.

2.6. Serum and tissue genistein analysis

Isoflavone extraction and analysis of serum samples using liquid chromatography-mass spectrometry (LC-MS) had been described previously [34,35]. Uterine tissues were homogenized in ammonium acetate (1 M, pH 7.4), and homogenates were hydrolyzed with hydrochloric acid at 100°C for 30 min. The acidified homogenates were extracted with hexane, and the resultant aqueous phase further extracted with ethyl acetate. The ethyl acetate fractions were dried under nitrogen at 50°C, reconstituted in 50% methanol/water containing a known amount of biochanin A as internal standard, and isoflavone content was determined using LC-MS. Isoflavones were detected using a PE Sciex API 100 Mass Spectrometer by negative single-ion monitoring. The assay detection limit was 0.03 ng.

2.7. Data analysis

Results are expressed as means±S.E.M. Differences between the two groups were determined by t test, and among diets, by one-way ANOVA. P values $<.05$ were considered statistically significant.

3. Results

3.1. Uterine growth

To evaluate the effects on uterine growth of lifetime exposure to SPI$^+$ and GEN, relative to CAS diet, uteri from normally cycling, young adult rats (PND50) were analyzed for luminal epithelium height, endometrial gland numbers, myometrial thickness and proliferation status. Dietary exposure to SPI$^+$ and GEN did not affect uterine luminal epithelium height (Fig. 1, top panel), uterine myometrial thickness (Fig. 1, middle panel) and uterine gland numbers (Fig. 1, bottom panel), relative to CAS diet. Further, proliferation (measured by immunohistochemical staining for PCNA) of endometrial glandular epithelial (GE), luminal epithelial (LE) and stromal (ST) cells did not differ among the diet groups (Fig. 2A). Representative uterine sections from CAS-, SPI$^+$- and GEN-fed rats, stained for PCNA, are shown in Fig. 2B.

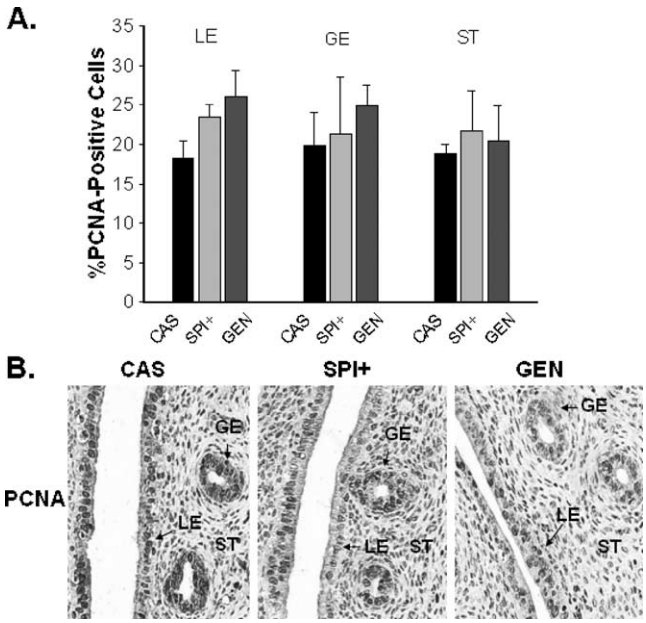

Fig. 2. Proliferation of major cell types in uteri of PND50 rats fed CAS±GEN or SPI$^+$ diets. (A) PCNA immunostaining is expressed as a percentage of the total number of cells counted. Data (mean±S.E.M.) were obtained from four randomly chosen fields (×200 magnification) per slide per PND50 rat ($n=4$ rats per diet group). (B) Representative uterine sections demonstrating PCNA immunoreactivity are shown for each diet group. GE, glandular epithelium; LE, luminal epithelium; ST, stroma.

Uterine GEN levels were measured, after tissue acid extraction, by LC-MS chromatography to determine tissue accumulation of GEN. Uteri of rats fed GEN had approximately 50% higher GEN content than those fed SPI$^+$ (0.63±0.24 vs. 0.33±0.11 nmol/g, respectively), although this difference did not reach statistical significance (Table 1).

3.2. Uterine apoptotic index

The numbers of TUNEL-positive cells were evaluated in uterine GE, LE and ST cells of rats fed CAS (control), SPI$^+$ or GEN diets (Fig. 3). The mean values of TUNEL-positive GE cells were as follows: CAS=1.5±0.3% (range 0.8–3%), SPI$^+$=5.4±2.8% (range 0.2–20.1%) and GEN=5.3±1.3% (range 3–12%). For LE cells, a mean value of 7.7±1.9% (range 1.7–10.9%) was observed for CAS, compared to 13.7±5.0% (range 0.5–30%) and 11.3±5.3% (range 0–28%) for SPI$^+$ and GEN, respectively. The mean values of TUNEL-positive ST cells were similar for all diet groups (CAS=0.65±0.17%; SPI$^+$=0.58±0.11%; GEN=0.43±0.14%). Among the three major endometrial cell types, only GE cells in uteri of GEN-fed rats had significantly increased apoptosis (P=.002) over that of the control (CAS) diet (Fig. 3).

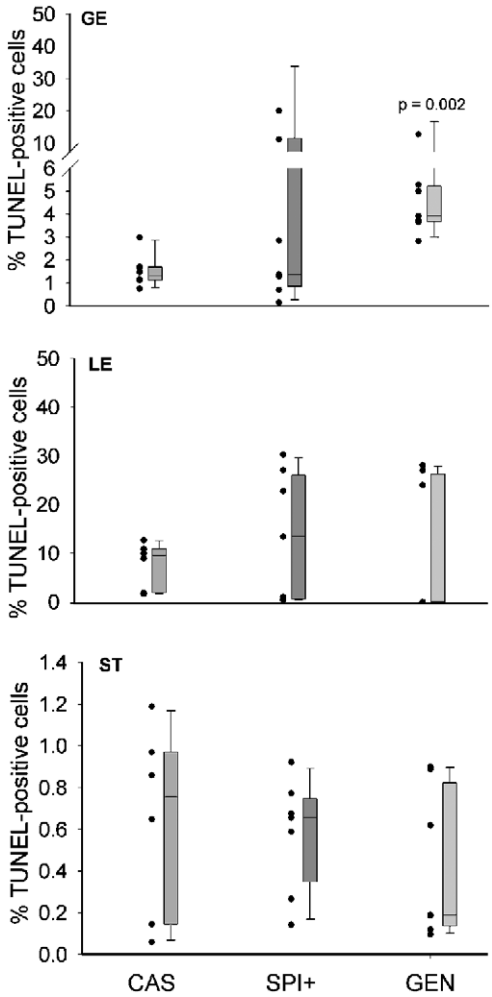

Fig. 3. Effect of diet on numbers of TUNEL-positive cells in uterine endometrium of PND50 rats. Results (shown as individual values for each animal and as bar graphs for mean±S.E.M.) are expressed as % of TUNEL-positive cells from an average of 500 total cells counted from three randomly selected fields (×200 magnification) per slide. Tissue sections (n=2 per animal) from six to seven animals per diet group (CAS, SPI$^+$, CAS+GEN) were analyzed. Top panel: GE, glandular epithelium; middle panel: LE, luminal epithelium; bottom panel: ST, stroma. P=.002, relative to CAS.

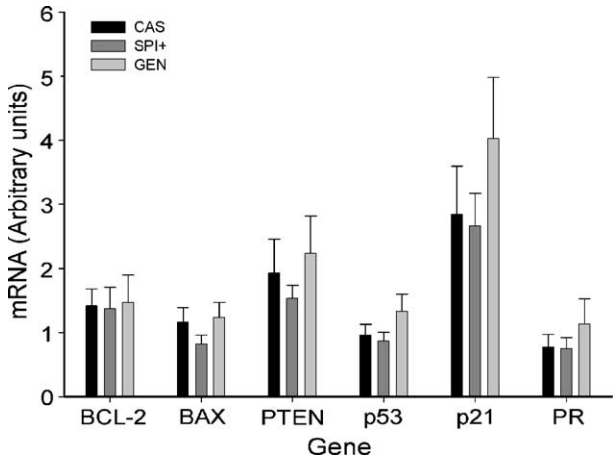

Fig. 4. Expression of growth-associated genes in uteri of PND50 rats fed CAS±GEN or SPI$^+$ diets. Levels of RNA transcripts (mean±S.E.M.; n=10 rats per diet group) were measured by quantitative RT-PCR and normalized to the control gene 18S rRNA. No differences in gene expression were observed among the diet groups.

To determine whether the range of apoptotic indices observed in uterine LE and GE cells within each diet might be correlated with variable serum steroid hormone concentrations, E and P levels were measured. The serum levels of E (pg/ml) were as follows: CAS=56.23±6.36, SPI$^+$=55.49±5.12 and GEN=40.03±8.92. The serum levels of P (ng/ml) were as follows: CAS=12.89±1.28, SPI$^+$=15.69±2.99 and GEN=14.80±2.37. There was no difference in E and P levels (P>.1) as a function of diet, consistent with normal estrous cycle duration and synchronous cyclicity among all diet groups (data not shown).

3.3. Uterine gene expression

To further evaluate the effects of diet on proliferation and apoptosis [36–38], the relative uterine transcript levels for the tumor suppressors PTEN, p53 and p21, and for the apoptotic-associated proteins Bcl2, Bax and PR, were determined. No differences were observed in the expression of all evaluated genes with dietary SPI$^+$ or GEN exposure, relative to CAS (Fig. 4).

3.4. In vitro effects of GEN on cellular apoptosis

To examine whether the modest increase in apoptosis in uterine glandular epithelium of rats exposed to dietary GEN (Fig. 3) was correlated with increased circulating GEN levels, serum from PND50 rats fed CAS, GEN or SPI$^+$ was analyzed for total GEN concentrations, following previously

described procedures from this group [34,35]. Levels of total GEN were higher in serum from rats fed GEN-supplemented CAS diet (1.47±0.04 μM) than in those fed SPI$^+$ (0.44±0.10 μM) or CAS alone (undetectable) (Table 1).

To evaluate whether the pro-apoptotic activity of GEN diet in vivo can be recapitulated by GEN serum in vitro, serum from CAS- and GEN-fed rats was added at a final concentration of 10% to Ishikawa endometrial carcinoma cells, and the number of apoptotic cells was counted 24 h later. For comparison, exogenously added GEN was evaluated for similar effects at two concentrations: that found in vivo in serum of rats fed GEN (2μM) and that added to Ishikawa cells when GEN serum was used at 10% final concentration (0.2 μM). The percentage of apoptotic cells tended to be higher in cells treated with serum from GEN-fed rats than from CAS-fed rats, although this difference did not reach statistical significance (P=.10) (Fig. 5A). Exogenously added GEN at 0.2 and 2 μM significantly increased the number of TUNEL-positive cells (P=.001) to the same level, while estradiol-17β at 0.1 μM (P<.05) decreased cell apoptosis, when compared to vehicle alone (Fig. 5B).

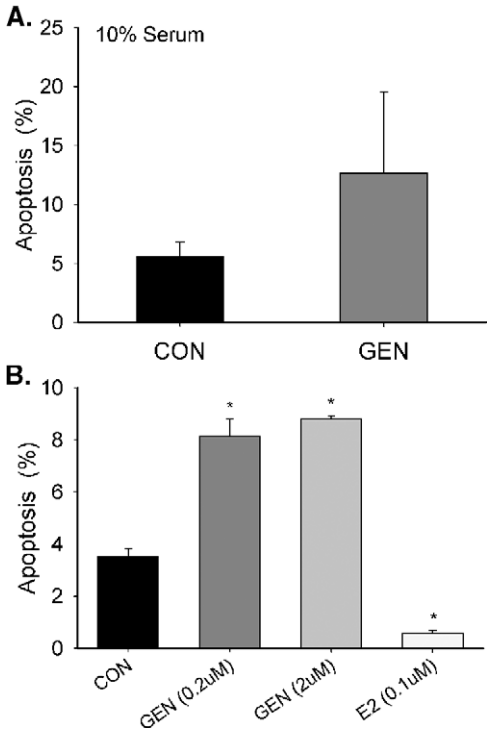

Fig. 5. In vitro effects of serum isolated from PND50 rats fed CAS±GEN or of exogenous GEN and estradiol-17β on human Ishikawa endocarcinoma cells. (A) Subconfluent Ishikawa cells were incubated for 24 h with pooled sera (10% final concentration) from PND50 rats fed CAS (n=6 rats) or CAS+GEN (n=6 rats), and apoptotic cells were visualized using fluorescein-labeled TdT reagent. Apoptotic index (% apoptosis) is expressed as a percentage of cells showing immunofluorescence over the total number of cells counted (average of 200–300 per given field). Results are mean (±S.E.M.) from n=3 independent experiments, with two replicates carried out per experiment. CON, sera from CAS-fed PND50 rats; GEN, sera from CAS+GEN-fed PND50 rats. (B) Ishikawa cells were treated with vehicle alone (control, CON), genistein (GEN, 0.2 or 2 μM) or estradiol-17β (E2, 0.1 μM), and analyzed for numbers of apoptotic cells following the protocol described in (A). * P<.05, relative to control. Results are mean (±S.E.M.) from n=2 independent experiments, with two replicates carried out per experiment.

4. Discussion

The present study evaluated the consequence of dietary exposure to soy or GEN supplement beginning at GD4 and continuing to the early adult stage (PND50), a regimen mimicking the temporal exposure of Asian women to soy-associated GEN, on uterine proliferation, apoptosis and gene expression in Sprague–Dawley rats. Results show that dietary intake of GEN, either as a component of SPI$^+$ or as supplement, elicited no or minimal effects, respectively, on uterine parameters that are considered to be intermediate markers of cancer risk [18,36–38]. Moreover, similar to its pro-apoptotic activity for breast cancer cells [39,40], exogenously added GEN increased the frequency of apoptosis in endometrial carcinoma cells of glandular origin (Ishikawa cells), and this effect was achieved at concentrations (0.2 and 2 μM) found in serum of rats fed soy or supplemental GEN. In a previous study, we showed that rats fed diets of CAS and SPI$^+$ over their lifetime did not differ in litter sizes for F1 and F2 generations [18]. Taken together with the present findings, these results suggest that GEN as part of a normal soy diet does not adversely affect the uterine phenotype of young adult females, and that supplemental GEN, by enhancing apoptosis in endometrial glandular epithelium in vivo, may confer protection against uterine carcinoma.

The present study is the first to assess in parallel the long-term effects of exposure to soy isoflavone ingested at two dietary contexts, as part of soy foods and as a supplement, beginning at the earliest stage of development (e.g., embryonic stage). Several possibilities may underlie the differences between our findings and those of other studies that demonstrated the negative consequences to uterine health of exposure to GEN in vivo [10–12,16,41]. As bioavailability of injected GEN is significantly greater than oral GEN [42], subcutaneous injection of the pure isoflavone could lead to a rapid elevation of its serum levels, resulting in an acute uterine response that is not otherwise attained when GEN is processed by the gut microflora. Moreover, GEN crossing the placenta may have a priming action in the fetus during the early stages of its uterine development, and which may be protective against additional estrogenic insult postnatal. In this regard, GEN has been demonstrated to act as a weak estrogen, but can antagonize estrogenic activity in the presence of estrogen in human endometrial stromal and glandular cells [43]. Further, chronic exposure to dietary soy or GEN past reproductive maturity may be necessary for detection of possible risks. Although Asian women on regular soya diet since early adolescence had no reported increased incidence of endometrial dysfunction [18–20], the negative effects of soy extracts and associated isoflavones had been described

for postmenopausal women, although this remains controversial [44,45] and requires further evaluation. Hence, the developmental stage, dose and route of exposure can markedly affect the degree of the uterine response, and these factors should be carefully considered when interpreting in vivo studies for dietary recommendations.

Genistein has been shown to induce apoptosis, a mechanism that is presumed to underlie, in part, the anticancer protective effects of soya diets containing GEN [39,40]. In the present study, we showed that dietary intake of exogenous GEN increased the apoptotic index in uterine glandular epithelium, but not in luminal epithelium or stroma. This effect of GEN was not mimicked by SPI^{+}, which is likely attributable to the higher uterine and serum GEN content in rats fed CAS+GEN. The basis for the different levels of GEN in vivo for the two diets is not clear at present given the comparable amounts of GEN aglycone provided in both diets, although this may be related to the distinct pharmacokinetics of protein-associated vs. free GEN. It is not known either why glandular epithelium, among the three major uterine cell types, was most responsive to GEN in vivo; however, the apoptotic activity of GEN in glandular epithelium was recapitulated in vitro using the Ishikawa cell line. Consistent with the limited apoptotic induction in this uterine compartment, there was a lack of notable changes in the uterine expression of multiple genes involved in the apoptotic pathway. Of particular note is the absence of a GEN effect on the gene expression of the tumor suppressor PTEN, whose activity is important for endometrial growth regulation in vivo and whose deregulated expression occurs in the earliest stages of endometrial carcinogenesis [46,47]. Although we did not evaluate the expression of other anticancer and/or estrogenic (e.g., pS2, C3) markers in the present study, the collective data predict a similar absence of diet effects on these genes' expression.

Results from the in vitro studies using Ishikawa cells suggest that dietary GEN supplement, by increasing serum GEN concentrations to "threshold concentrations" requisite for apoptotic induction, may confer protection against uterine carcinoma. Because normal intake of GEN as part of a normal soya diet did not raise the circulating levels of GEN to that attained with dietary GEN and, predictably, did not affect the status of any uterine parameter relative to the control diet devoid of GEN, these findings suggest that the two dietary contexts evaluated here can be uniquely utilized for their potential health benefits. Dietary intake of GEN in soy foods may be relevant to the prevention of adult chronic diseases such as cardiovascular disease and mammary cancer [48,49], while, as a supplement, should be exploited as a preventative strategy or therapy for uterine carcinoma. Nevertheless, it is worth noting that, in vitro, we did not observe a dose-dependent effect of exogenously added GEN on Ishikawa cell apoptosis, in contrast to that found in mammary glands in vivo. Moreover, estradiol-17β decreased apoptosis in these cells, in opposition to the effects elicited by exogenous GEN and by serum from rats fed GEN. These observations may reflect the following: (1) GEN undergoes in vivo modification to distinct forms that do not occur in vitro and which can influence GEN bioactivity, (2) underlying stromal cells contribute to epithelial cell response to GEN in vivo and (3) GEN does not mimic estrogen in mediating cell apoptosis at the concentrations tested. Further studies to evaluate these possibilities will provide important insights on the molecular mechanisms of GEN action related to its health benefits.

In summary, we conclude that dietary exposure to soy-associated GEN does not alter the uterine phenotype of normally cycling, young adult rats that could be predictive of increased risk for uterine hyperplasia and adenocarcinoma at later adult life. Further, supplemental intake of GEN has the potential for endocarcinoma prevention via its induction of uterine cell apoptosis. Thus, our results and those obtained from human epidemiological studies on the health benefits of soy [2–4,19] suggest that the efficacy of GEN, when taken as part of a regular soy diet, in the prevention of adult chronic diseases, is not necessarily compromised by putative adverse effects on uterine health.

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