Original Research

Soy protein isolate feeding does not result in reproductive toxicity in the pre-pubertal rat testis

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Impact statement

Soy protein isolate (SPI) is the sole protein used to make soy-based infant formulas. SPI contains phytoestrogens, which are structurally similar to estradiol. These phytoestrogens, daidzein, genistein, and equol, fit the definition of endocrine-disrupting compounds, and at high concentrations, have estrogenic actions resulting in reproductive toxicity in the developing male, when provided as isolated chemicals. However, few animal studies have examined the potential estrogenicity of SPI as opposed to pure isoflavones. In this study, SPI feeding did not elicit an estrogenic response in the testis nor any adverse outcomes including reduced testicular growth, or androgen production during early development in rats when compared to those receiving estradiol. These findings are consistent with emerging data showing no differences in reproductive development in males and female children that received breast milk, cow's milk formula, or soy infant formula during the postnatal feeding period.

Abstract

The isoflavone phytoestrogens found in the soy protein isolate used in soy infant formulas have been shown to have estrogenic actions in the developing male reproductive tract resulting in reproductive toxicity. However, few studies have examined potential estrogenicity of soy protein isolate as opposed to that of pure isoflavones. In this study, we fed weanling male Sprague-Dawley rats a semi-purified diet with casein or soy protein isolate as the sole protein source from postnatal day 21 to 33. Additional groups were fed casein or soy protein isolate and treated s.c. with 10 μg/kg/d estradiol via osmotic minipump. Estradiol treatment reduced testis, prostate weights, and serum androgen concentrations ($P < 0.05$). Soy protein isolate had no effect. Estradiol up-regulated 489 and down-regulated 1237 testicular genes >1.5-fold ($P < 0.05$). In contrast, soy protein isolate only significantly up-regulated expression of 162 genes and down-regulated 16 genes. The top 30 soy protein isolate-up-regulated genes shared 93% concordance with estradiol up-regulated genes. There was little overlap between soy protein isolate down-regulated genes and those down-regulated by estradiol treatment. Functional annotation analysis revealed significant differences in testicular biological processes affected by estradiol or soy protein isolate. Estradiol had major actions on genes involved in reproductive processes including down-regulation of testicular steroid synthesis and expression of steroid receptor activated receptor (Star) and cytochrome P450 17α-hydroxylase/(Cyp17a1). In contrast, soy protein isolate primarily affected pathways associated with macromolecule modifications including ubiquitination and histone methylation. Our results indicate that rather than acting as a weak estrogen in the developing testis, soy protein isolate appears to act as a selective estrogen receptor modulator with little effect on reproductive processes.

Keywords: Soy protein isolate, isoflavones, estrogenicity, testes, reproductive toxicity

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Introduction

Soy foods such as tofu and tempeh have been a mainstay of traditional Asian diets for thousands of years. More recently, soy protein isolate (SPI) has become ubiquitous in Western diets as meat substitutes, additives to processed foods, and as the sole protein source in soy infant formulas.[1,2] SPI is complex mixture consisting of hundreds of phytochemicals in addition to isoflavones, namely

genistein and daidzein, bound to potentially bioactive proteins and peptides.[3] The isoflavones together with the microbiota-derived daidzein metabolite equol can bind to and activate estrogen receptors (ERs). In this regard, these compounds fit the definition of endocrine disrupting compounds (EDCs) which have the potential to interfere with reproductive development and fertility.[4] The potential adverse health consequences of EDC exposure are the subject of increasing concern among the endocrine and toxicology research community. In 2011, these concerns led to a reevaluation of the safety of soy infant formula by a review panel from the Center for the Evaluation of Risks to Human Reproduction (CERHR) established by the National Toxicology Program (NTP) and the National Institute for Environmental Health Sciences (NIEHS). However, recommendations regarding the reproductive safety of soy formula by the NTP and NIEHS have been inconclusive due to a paucity of experimental and human clinical data.[5]

Many cells in the male reproductive axis express ERα and ERβ in different proportions. This includes testicular Sertoli, Leydig, and germ cells.[6,7] Exposure to ER agonists during early male development is well known to interfere with growth and development of the reproductive tract, suppress androgen production, and reduce fertility.[4,8] For example, the synthetic estrogen diethylstilbestrol (DES) reduces testis and prostate size, inhibits testosterone synthesis, alters seminiferous tubule morphology, and disrupts spermatogenesis as a result of increased apoptosis.[9–11] Increased estrogen production as a result of aromatase over-expression produces infertility and increases Leydig cell tumor incidence in adulthood.[12] Conversely, treatment of mice with the ER antagonist ICI 182,780 and genetic ablation of ERα increases Leydig cell androgen synthesis as a result of increased expression of steroidogenic acute regulatory protein (Star); cytochrome P450 17α-hydroxylase/(Cyp17a1) and 17β-hydroxysteroid dehydrogenase (17β-Hsd3).[13]

With respect to soy isoflavones, Weber *et al.*[14] reported that consistent with ER activation, feeding adult rats with a diet enriched with pure soy isoflavones significantly reduced prostate weight and plasma androgen concentrations. Wisniewski *et al.*[15] reported that genistein exposure through gestation and lactation demasculinized the rat reproductive system by reducing testis size and testosterone concentrations, and disrupted reproductive behavior in adulthood.[15] More recently, Meena *et al.*[16] have reported that prenatal genistein exposure reduced fertility in adult male rats associated with reduced reproductive organ weights, a deterioration of testicular architecture, dose-dependent reductions in sperm production, epididymal sperm density and quality, and decreased plasma testosterone associated with decreases in activity of 3β and 17β-hydroxysteroid dehydrogenase activities.[16] In male mice, genistein treatment in adults has been reported to result in disrupted spermatogenesis as the result of up-regulation of miR-20a and its target gene Limk1.[17] *Ex-vivo* studies have reported that genistein and daidzein inhibited Leydig cell steroidogenesis and more recently, this effect has been associated with signaling via ERα.[18–20] In contrast, we have reported a lack of endocrine disruption, no effects on development of male reproductive organs or effects on fertility in rats fed SPI throughout development.[1,2] Moreover, we have observed dramatically different gene expression profiles in liver, bone, mammary gland, and uterus of adult and pre-pubertal rats after feeding SPI relative to those elicited by 17β estradiol (E2).[21–25] These data suggest that the endocrine effects of purified isoflavones relative to feeding SPI are quite different. The current study was designed to determine if feeding SPI, the sole protein source in soy infant formula to pre-pubertal male rats has different effects than estrogens on male reproductive development during a developmental window corresponding to human neonatal and early childhood development. We directly compared the effects of feeding SPI and 17β-E2 treatment alone or in combination on sex organ weights, endocrine profile, and global testicular gene expression profiles in male rats treated from weaning on post-natal day (PND) 21 until prior to puberty on PND 33.

Materials and methods

Animal care and experimental design

Pregnant Sprague-Dawley rats (Harlan Industries, Indianapolis, IN) were fed semi-purified AIN-93G diets containing 20% casein protein and with corn oil replacing soybean oil from gestational day 4 as previously described.[26] Pups continued to be fed casein AIN-93G throughout lactation. On PND 21, the pups were separated by sex and implanted with Alzet, 2002 min-osmotic pumps s.c. (Alza, Mountain View, CA). At this time, male pups ($n = 10$/group) were assigned to one of four isocaloric treatment groups: fed casein AIN-93G and infused with polyethylene glycol (PEG) vehicle (CAS); fed casein AIN-93G diet and infused s.c. with E2 17β-E2 at 10 μg/kg/d (CAS + E2); fed AIN-93G diets made with SPI in place of casein (SPI) or fed AIN-93G diets made with SPI and infused s.c. with E2 at 10 μg/kg/d (SPI + E2). SPI had 3.21 g/kg total isoflavones, including 1.87 g/kg genistein-containing compounds equivalent to 1.08 g/kg genistein aglycone equivalents and 1.22 g/kg daidzein-containing compounds corresponding to 0.69 g/kg daidzein aglycone. Treatment ended on PND 34 when rats were killed at 0800 h by anesthetization with Nembutal (100 mg pentobarbital i.p.) followed by decapitation. Blood was collected and serum frozen at −20°C for analysis. In addition, testes and prostate were weighed and flash frozen in liquid nitrogen, then stored frozen at −80°C until use. All animal protocols were approved by the Institutional Animal Care and use Committee at the University of Arkansas for Medical Sciences.

Serum isoflavone and hormone concentrations

Serum total conjugated isoflavones and free isoflavone aglycones were extracted and measured by LC-MS as described previously.[27] Serum hormone concentrations were measured according to manufacturer's instructions: testosterone (T), E2, and progesterone were measured by radioimmunoassay (DSL-4100; DSL-4800 and DSL-3400;

Beckman Coulter, Indianapolis, IN). Androstenedione was measured by ELISA (ab108672, AbCam, Cambridge, MA). Dihydrotestosterone (DHT), dehydroepiandrostenedione sulfate (DHEA-S), and estrone (E1) and luteinizing hormone (LH) were measured by ELISA (MBS701006, MBS9302097, MBS105877, MBS007779; MyBioSource, San Diego, CA).

Testis microarray set

Microarray analysis was performed on testicular preparations. In brief, 8 μg of purified mRNA was extracted from frozen testicular tissue using TRI reagent (Molecular Research Center, Cincinnati, OH) and cleaned up with an RNeasy Mini Kit (Qiagen, Valencia, CA). RNA was processed according to manufacturer's instructions (one-cycle cDNA synthesis kit and GeneChip IVT labeling kit; Affymetrix, Santa Clara, CA). The resulting cRNAs from rat testis ($n = 3$ different pools from each treatment group, each containing mRNA extracted from 3 or 4 individuals) were hybridized to an Affymetrix GeneChip Rat Genome 230 2.0 following the manufacturer's protocol (Affymetrix). The probe array was washed and stained using the Affymetrix kit in a GeneChip fluidics station 450 and scanned using GeneChip Scanner 3000. Data on the probe level intensities were extracted using GeneChip operating software obtained from Affymetrix and CEL data files were generated. Files were deposited in the gene expression omnibus (GEO) repository at the National Center for Biotechnology Information (http:/www.ncbi.nlm.nih.gov/geo/) under GEO accession no. GSE94392. AffyQReport software was utilized to verify that the quality metrics for the microarrays were acceptable. A pairwise distance analysis was utilized to cluster the arrays by expression levels. Phenotype clustering revealed clustering between the CAS and SPI groups, whereas the E2 and E2 + SPI groups clustered separately (data not shown), and consistent with previous results.[22–24,28] Probe level diagnostics (affyPLM) was utilized to determine standard error estimates for each gene on every array.[29,30] Background correction and quantile normalization were conducted using robust multi-array analysis.[31] A non-specific filter was set to eliminate probes whose variances across samples were below 0.5 since these genes are less likely to provide biological information. Differential analysis of gene expression between treatments was analyzed using the following linear model

$$Yij = \mu j + \beta^{j}{}_{E2} x_{E2} + \beta^{j}{}_{soy} x_{soy} + \beta^{j}{}_{E2.soy} x_{E2} x_{soy} + \varepsilon ij$$

where Yij is the expression level for transcript j in sample i ($i \varepsilon \{1,\ldots 18\}$; μj is the expression level of the transcript j for the control (CAS) group; x_{E2} is a discrete variable that takes the value 1 when E2 treatment is in addition to diet and zero otherwise; x_{soy} is a discrete variable that takes the value 1 when the diet is soy and zero otherwise; β^{j}_{E2} represents the effect of E2 on transcript j; β^{j}_{soy} represents the effect of soy diet on transcript j; $\beta^{j}_{E2.soy}$ represents the interaction term that quantifies changes for changes for the combination of soy diet and E2 for transcript j. εij is a normally distributed error with zero mean and variance σ^2_j. Estimates of linear models for each transcript were determined using the *limma* package. Significant differences in mRNA transcript expression had at least a 1.5-fold change from CAS and an FDR-adjusted P-value of 0.05. Functional analysis on gene expression was performed with DAVID v.3.6,[32] and with the plugin of BinGO[33] for Cytoscape v.3.6.[34] The core ontology was downloaded from the gene ontology consortium. Data in Figure 2 were developed with Ciros v. 0.69–6.[35]

Microarray validation by real-time quantitative PCR. Total RNA (1 μg) was reverse-transcribed using iSCRIPT cDNA synthesis kit (Bio-Rad, Hercules, CA), following the manufacturer's instructions. cDNA samples were amplified using previously described conditions.[24] Quantitative real-time PCR (qRT-PCR) was performed on genes that were of particular interest to this study: Fabp4 F 5′-GTGTCACGGCTACCAGAATT TA-3′, R 5- CGGGACA ATACATCCAACAGAG-3′; Car3 F 5′-GCTCAGCTTCAC CTGGTTCAC-3′, R 5′ AATGCCAACCACAGCAATCC-3′; Hivep1 f 5′- CAAAGTCATCCAAGGGCAAAGGCA-3′, R 5′-AGGGCAATGACTTCTGTCTCAGCA-3′; Myh6 F 5′- C CATCCTCATCACTGGAGAAT C-3′, R 5′-GGTGCCCTTG TTTGCATTAG-3′; Star F 5′-TGCTCAGCATTGACCTCAA GGGAT-3′, R 5′-TTTCGAAGGTGATTGGCAAACTCC-3′; Cyp17a1 F 5′-GGTGCCCAGACCACAATTTA-3′, R 5′-CT TTACCACAGAGGCAGAAGTC-3′; Srp14 F 5′-TAACAA GTGCATCAGAGACGGGC-3′, R 5′-TGGAATGGAAGCC AGACACCAGC-3′. Gene expression was analyzed using the $2^{-\Delta\Delta ct}$ method relative to *Srp14* gene amplification.

Western blotting. Tissue lysates were prepared from 100 mg of individual frozen testis tissue homogenized in RIPA buffer. Proteins (20 μg) were separated by SDS-polyacrylamide gel electrophoresis using standard methods. Blotted proteins were incubated with Cyp17al anti-rabbit monoclonal antibody (LS-B6749, LifeSpan Biosciences, Seattle, WA) using a 1:1000 dilution, or incubated with Star anti-rabbit polyclonal antibody (Ab96637, Abcam, Cambridge, MA) using a 1:1000 dilution. Secondary antibodies were diluted (1:5000 to 1:10000) and incubated at room temperature before chemiluminescence detection. Quantitation of relative expression was based on densitometric analysis adjusted for differences in loading of total protein as determined by amido black staining of the entire lane.

Statistical analysis. Data analysis of the microarray data is described above. Experimental outcomes were summarized as mean ± standard error (SEM). A two factor, diet (CAS/SPI), E2 treatment (yes/no) analysis of variance (ANOVA) was used for analysis of body weight, tissue weights, serum hormone concentrations, and for real-time RT-PCR gene expression and Western immunoquantitation of protein expression to test main effects and interactions. Pair-wise comparisons using Tukey–Kramer *post hoc* analysis were performed to identify groups whose means differed significantly $P < 0.05$. Statistical analyses were

performed using the Stata statistical package version 12.0 (Stata Corporation, College Station, TX, USA).

Results

Serum isoflavones

Serum concentrations for different isoflavones and their metabolites following feeding of SPI and SPI + E2 diets are shown in Table 1. Genistein, daidzein, glycetin, and their metabolites dihydrogenistein (DHG), dihydrodaidzein (DHD), equol, and O-desmethylangolansin (ODMA) were undetectable in rats fed AIN-93G made with casein but were all present in rats fed the same diet where SPI was substituted for casein. In the SPI and SPI + E2 groups, total isoflavone concentrations were ~5 μM, a concentration similar to levels observed in soy formula-fed infants.[36] Moreover, greater than 95% of the isoflavones were found in their conjugated form (Table 1). Interestingly, we did observe a trend for an increase in the sum of free isoflavones in rats receiving the SPI + E2 diet relative to those receiving the SPI diet ($P = 0.06$, Student's *t*-test). This increase was associated with significantly higher genistein and daidzein serum concentrations in the SPI + E2 vs. SPI group. In the SPI + E2 group, we also observed a significant decreased in circulating daidzein conjugates relative to the SPI group; however, there was no difference in the sum of total conjugates between the two diet groups.

Body weight and tissue weights

Both feeding of SPI and E2 treatment resulted in reduced body weight relative to the CAS group ($P < 0.05$); moreover, this effect was additive with the lowest body weight observed in the SPI + E2 group ($P < 0.05$ vs. SPI and vs. E2) (Table 2). E2 treatment also significantly reduced the weight of both male sex organs relative to the CAS group (Table 2). In contrast, both absolute and relative weights of the testis and prostate were unaffected by SPI feeding

Table 1. Serum isoflavone concentrations (μM) in SPI-fed male rats at PND 33.

	Diet					
	AIN-93		SPI		SPI+E2	
	Free	Total	Free	Total	Free	Total
DHG	ND	ND	<0.001	0.09 (0.02)	<0.001	0.06 (0.01)
DHD	ND	ND	<0.001	0.11 (0.01)	<0.001	0.04 (0.01)
Equol	ND	ND	0.023 (0.009)	3.31 (0.31)	0.031 (0.009)	4.26 (0.43)
Glycitein	ND	ND	0.003 (0.001)	0.03 (0.001)	0.004 (0.006)	0.02 (0.003)
Genistein	ND	ND	0.013 (0.001)[a]	0.78 (0.11)	0.031 (0.001)[b]	0.57 (0.61)
Daidzein	ND	ND	0.010 (0.001)[a]	0.75 (1.04)[a]	0.031 (0.004)[b]	0.33 (0.03)[b]
O-DMA	ND	ND	0.005 (0.001)	0.70 (0.08)	0.002 (0.001)	0.19 (0.04)
SUM	ND	ND	0.058 (0.011)[a]	5.8 (0.45)	0.10 (0.016)[a,b]	5.50 (0.52)

Note: Different superscript letters indicate groups are significantly different (P<0.05). Data are means (SE) for $n = 8$ male rats receiving AIN-93 diets containing casein (AIN-93), soy protein (SPI) or soy protein plus 10 μg/kg/d estradiol (SPI + E2). Serum values for isoflavones, dihydrogenistein (DHG), dihydrodaidzein (DHD), equol, glycitein, genistein, daidzein, and O-desmethylangolansin (O-DMA) were determined by LC/MS as previously described25. Total includes free aglycone + glucuronide and sulfate conjugates. Statistical differences were determined by Student's t-test analysis ($P < 0.05$), for differences between free and total isoflavones, SPI vs. SPI + E2.
SPI: soy protein isolate; PND: post-natal day.

Table 2. Physiological and endocrine values.

	CAS	CAS+ E2	SPI	SPI+E2	*P*
Body weight (g)	152.00 (5.35)[c]	139.6 (4.61)[b]	135.00 (4.06)[b]	120.50 (3.00)[a]	<0.001
Testis weight (g)	1.25 (0.15)[b]	0.75 (0.10)[a]	1.20 (0.05)[b]	0.65 (0.09)[a]	<0.001
Testis weight (%)	0.82 (0.10)[b]	0.54 (0.07)[a]	0.88 (0.04)[b]	0.54 (0.07)[a]	<0.001
Prostate weight (g)	0.12 (0.02)[b]	0.05 (0.02)[a]	0.12 (0.01)[b]	0.06 (0.01)[a]	<0.001
Prostate weight (%)	0.08 (0.01)[b]	0.05 (0.01)[a]	0.09 (0.01)[b]	0.04 (0.01)[a]	<0.001
Pregnenolone (ng/ml)	2.59 (0.50)	4.00 (0.62)	3.00 (0.21)	3.09 (0.30)	0.175
Progesterone (ng/ml)	1.44 (0.36)	1.91 (0.38)	1.28 (0.25)	1.54 (0.34)	0.632
Androstenedione (ng/ml)	0.20 (0.03)[a,b]	0.26 (0.08)[b]	0.13 (0.05)[a]	0.13 (0.03)[a]	0.003
DHEA-S (ng/ml)	2.71 (0.66)[a]	10.40 (2.72)[c]	3.68 (0.37)[b]	8.21 (0.29)[c]	<0.001
Testosterone (ng/ml)	0.32 (0.13)[b]	0.03 (0.01)[a]	0.23 (0.09)[b]	0.01 (0.01)[a]	<0.001
DHT (ng/ml)	84.50 (26.9)[b]	41.91 (2.92)[a]	76.07 (13.00)[b]	37.40 (1.29)[a]	0.012
E2 (pg/ml)	5.52 (0.63)[a]	12.75 (2.19)[b]	6.22 (0.22)[a]	17.79 (1.76)[c]	<0.001
E1 (pg/ml)	20.90 (1.87)[a]	30.59 (1.63)[b]	27.2 (0.84)[b]	28.20 (1.74)[b]	0.002
LH (mlU/ml)	0.29 (0.07)	0.32 (0.11)	0.41 (0.11)	0.26 (0.04)	0.703

Note: Data are mean ± SEM for n = 10/group. ANOVA analysis was performed on log transformed data followed by Student–Newman–Keuls all pairwise multiple comparisons. Different superscript letters indicate groups are significantly different ($P < 0.05$) a < b < c.
SPI: soy protein isolate; CAS: casein; E2: estradiol; DHEA-S: Dehydroepiandrosterone-sulfate; DHT: dihydrotestosterone; E1: estrone; LH: luteinizing hormone.

relative to feeding CAS diets. Substitution of SPI for CAS had no significant additional effects on testis or prostate weights in the SPI + E2 group relative to E2 treatment alone.

Endocrine parameters

E2 treatment had no significant effect on serum pregnenolone, progesterone, or androstenedione concentrations. However, both serum testosterone and DHT were reduced by E2 treatment relative to CAS controls ($P < 0.05$). E2 treatment resulted in significant increases in both serum E1 and E2 ($P < 0.05$) (Table 2). E2 treatment also increased serum concentrations of the adrenal steroid DHEA-S in both the presence and absence of feeding SPI ($P < 0.05$). Substitution of SPI for CAS in the diet resulted in no effects on serum pregnenolone or progesterone but reduced androstenedione and significantly increased DHEA-S and E1 ($P < 0.05$) (Table 2). However, no effects were observed on serum concentrations of testosterone, DHT, or E2 (Table 2). Feeding of SPI in addition to E2 treatment had no effects on serum pregnenolone, progesterone, testosterone, DHT, and E1 relative to E2 alone. However, the combination did reduce serum androstenedione and further increased serum E2 levels relative to E2 alone ($P < 0.05$). E2 and SPI effects on sex steroid concentrations occurred independent of any effects on serum concentrations of LH.

Testicular gene expression profiles in SPI-fed, E2-treated, and SPI-fed/E2-treated prepubertal male rats

Testicular gene expression profiles differed significantly between E2-treated groups and the CAS or SPI-fed rats. Using a 1.5-fold cut off and FDR-adjusted P-value <0.05, E2-treatment resulted in significant changes in expression of 1726 mRNAs species relative to CAS controls (Figure 1). Of these, 489 genes were up-regulated and 1237 genes were down-regulated. In contrast, SPI feeding only changed expression of 178 genes (10.7% of E2-regulated genes). However, 151 of these (86%) overlapped with E2-regulated mRNAs; 162 SPI-regulated genes were up-regulated and 16 down-regulated. Interestingly, there was a 93% concordance between the top 20 E2 and SPI up-regulated genes with similar 2–5-fold induction (Tables 3 and 5). In contrast, expression of none of the top 20 E2 down-regulated genes was significantly affected by feeding SPI and the SPI down-regulated genes were either not regulated by E2 or were actually E2 up-regulated (Tables 4 and 6). The combination of SPI feeding and E2 treatment changed 2184 genes of which 688 were up-regulated and 664 down-regulated (Figure 1). Gene ontology analysis revealed significant differences in biological processes regulated by E2 and SPI (Figures 2 to 5). The top pathways regulated by E2 were

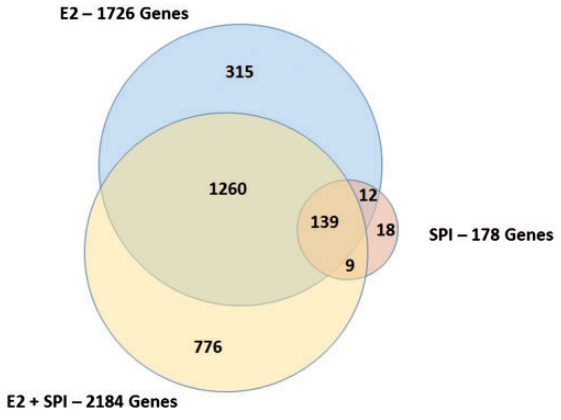

Figure 1. Overlap in testis mRNA profiles between E2, SPI, and E2 + SPI in male prepubertal rats. Number of significantly upregulated or down-regulated genes that are specific or in common to each treatment. (A color version of this figure is available in the online journal.)

Table 3. Top 20 E2 up-regulated genes in rat testis PND33.

Gene	Fold change (E2)	Fold change (SPI)	Fold change (E2 + SPI)
Car3	9.7	1.8	15.6
Sf3b1	5.6	3.0	6.5
Eprs	5.3	4.6	6.3
Garnl1	4.5	3.2	N.S.
Tm9sf3	4.0	3.7	4.5
Pbrm1	3.9	2.9	4.0
Bat2d1	3.8	2.6	N.S.
Mil1	3.5	N.S.	N.S.
RGD130829	3.4	2.5	N.S.
Zbtb20	3.2	2.7	3.9
Gpbp1	3.2	3.0	3.5
Myh6	3.2	N.S.	4.4
Atrx	3.1	2.0	2.6
Msi2	3.1	2.2	3.9
Rest	3.1	2.0	1.5
Syncrip	3.0	1.9	2.8
Sec63	2.9	2.1	1.6
Jam2	2.8	2.0	1.6
Wdr75	2.8	2.0	1.5
Ash1l	2.8	1.9	2.0

SPI: soy protein isolate; PND: post-natal day; E2: estradiol.

Table 4. Top 20 E2 down-regulated genes in rat testis PND33.

Gene	Fold change (E2)	Fold change (SPI)	Fold change (E2 + SPI)
Ces3	−6.5	N.S.	N.S.
Pah	−5.8	N.S.	−5.9
Tnnc1	−4.5	N.S.	−5.2
Pitx2	−4.2	N.S.	−4.0
RGD131187	−3.9	N.S.	−2.9
Atpb2	−3.8	N.S.	N.S.
RGD156315	−3.6	N.S.	−2.9
RGD131124	−3.6	N.S.	−3.0
RGD156242	−3.6	N.S.	N.S.
Fscn3	−3.5	N.S.	−3.5
Prm2	−3.4	N.S.	−2.5
Insl3	−3.4	N.S.	−4.0
RGD156323	−3.4	N.S.	−2.2
Agpat2	−3.3	N.S.	−2.7
Ankrd60	−3.3	N.S.	−2.5
Prkar2a	−3.3	N.S.	−2.9
Pwwp2b	−3.3	N.S.	−2.6
Arl9	−3.3	N.S.	−2.4
RGD130662	−3.2	N.S.	−2.4
Gpd2	−3.2	N.S.	−2.6

SPI: soy protein isolate; PND: post-natal day; E2: estradiol.

Table 5. Top 20 SPI up-regulated genes in rat testis PND33.

Gene	Fold change (E2)	Fold change (SPI)	Fold change (E2 + SPI)
Eprs	5.3	4.6	6.3
Tm9sf3	4.0	4.5	4.5
Garnl1	4.5	3.2	N.S.
Usp8	2.3	3.1	2.3
Ttk	2.7	3.0	3.1
Gpbp1	3.2	3.0	3.5
Sf3b1	5.5	3.0	6.5
Pbrm1	3.9	2.9	4.0
Pde8a	2.4	2.8	2.5
Zbtb20	3.2	2.7	3.9
Lrpprc	2.6	2.6	2.0
Bat2d1	3.8	2.6	N.S.
Usp42	2.2	2.6	2.5
RGD130829	−1.6	2.5	N.S.
Spag9	2.7	2.5	2.8
Gmps	2.0	2.5	2.0
Wdr47	2.0	2.5	−1.5
RGD130553	2.8	2.5	N.S.
Tlk2	1.9	2.4	2.3
Usp7	1.7	2.4	2.0

SPI: soy protein isolate; PND: post-natal day; E2: estradiol.

Table 6. Significantly SPI down-regulated genes in rat testis PND33.

Gene	Fold change (E2)	Fold change (SPI)	Fold change (E2 + SPI)
Asb18	−1.5	−1.8	−1.8
RT1-CES	N.S.	−1.8	N.S.
Hivep1	N.S.	−1.7	N.S.
Abcb1	N.S.	−1.6	N.S.
H2-Ea	N.S.	−1.6	N.S.
Nik	N.S.	−1.6	N.S.
Vpreb3	N.S.	−1.6	N.S.
Cd74	3.9	−1.6	N.S.
Ncr3	2.4	−1.6	−2.4
MGC11620	3.2	−1.6	N.S.
Ube2d1	2.6	−1.5	N.S.
Hp	3.8	−1.5	N.S.
RGD1308772	2.2	−1.5	−3.9
P2rx2	−1.6	−1.5	−2.6
Apba3	2.7	−1.5	N.S.

SPI: soy protein isolate; PND: post-natal day;E2: estradiol.

reproductive processes: gamete generation, spermatogenesis, sperm flagellum, and chromosome segregation and also histone methylation (Figure 2). Although some of these genes such as steroid hormone binding globulin (Shbg) were up-regulated, most of these genes, including Esr-1 (ERα itself) and the genes involved in androgen production including Star, Cyp17a1 and 17β-Hsd3, were significantly down-regulated indicating impairment of testicular function. SPI significantly regulated very few of these genes (Figures 2 to 4). In contrast, the major biological processes linked to SPI-regulated genes were in other E2-regulated pathways, in particular protein modification via ubiquitination and protein catabolism and in E2-indpendent regulation of chromatin via histone methylation (Figure 4). E2 and SPI-jointly upregulated genes included the ubiquitin specific proteases (deubiquitylating enzymes) Usp7, Usp8, and Usp42 (Tables 3 and 5). Usp7 is a direct antagonist of Mdm2 and stabilizes p53 in response to stress.[37] Usp8 regulates the epidermal growth factor receptor (Egrf) degradation and downstream MAP kinase signaling,[38] and Usp42 regulates spermatogenesis.[39] Other jointly E2 and SPI up-regulated testicular genes are involved in regulation of the cell cycle, including, glutamyl prolyl tRNA synthases (Eprs), Ttk protein kinase (Ttk), and Tousled-like kinase 2 (Tlk2).[40–42] SPI-specific down-regulated genes (Table 6) included H2-Ea and Cd74, components of the histocompatibility complex[43,44]: Map3 kinase 14, NFκB inducing kinase (Nik), and natural cytotoxicity triggering receptor 3 (Ncr3) which have immune regulatory roles.[45,46]

Verification of array analysis at mRNA and protein level

We have verified many of the changes in testicular gene expression at the level of individual mRNAs using real-time RT-PCR. These include up-regulation of mRNA encoding Fabp4 and Car3 in both E2 and SPI groups; down-regulation of Hivep1 mRNA by SPI; up-regulation of Myh6 mRNA in the E2 group and down-regulation of Star and Cyp17a1 mRNAs in the E2 group (Figure 5). However, concordance between the array data and real-time RT-PCR results was not absolute, possibly since many of the changes in gene expression were relatively small and the microarray data are based on analysis of $n = 3$ sets of pooled samples as opposed to analysis of samples from 10 individual animals. We verified the selective down-regulation of testicular Star and Cyp17a1 proteins by E2 but not SPI by Western immunoblotting (Figure 6).

Discussion

Exposure to estrogens during early development when endogenous estrogens are very low is well known to reduce androgen production, alter development of the male reproductive tract, and reduce male fertility.[6,8] As a result, concerns have been raised regarding potential demasculinization and reproductive toxicity in boys fed soy formula.[4,47,48] Few epidemiological or clinical studies have examined the long-term effects of soy foods on the male reproductive system.[4] In adults, meta-analysis shows no significant effects of soy protein or isoflavones on male reproductive hormones and the small number of studies on semen quality are contradictory.[4,49] In children, recent data from a longitudinal ultrasound study of breast, cow's milk formula, and soy-formula-fed infants (The Beginnings Study) demonstrated no significant diet effects on testis or prostate volumes or structural characteristics at age one year and five years.[50,51]

The goal of the current study was to test the hypothesis feeding SPI, the sole protein source in soy infant formula, during pre-pubertal development in males does not result in endocrine disruption consequent to ER activation. This hypothesis is based on (1) when compared to purified isoflavones, SPI is a food stuff composed of complex mixtures, i.e. proteins, peptides, and over 100 phytochemicals, all of which may have biological activity such that the SPI

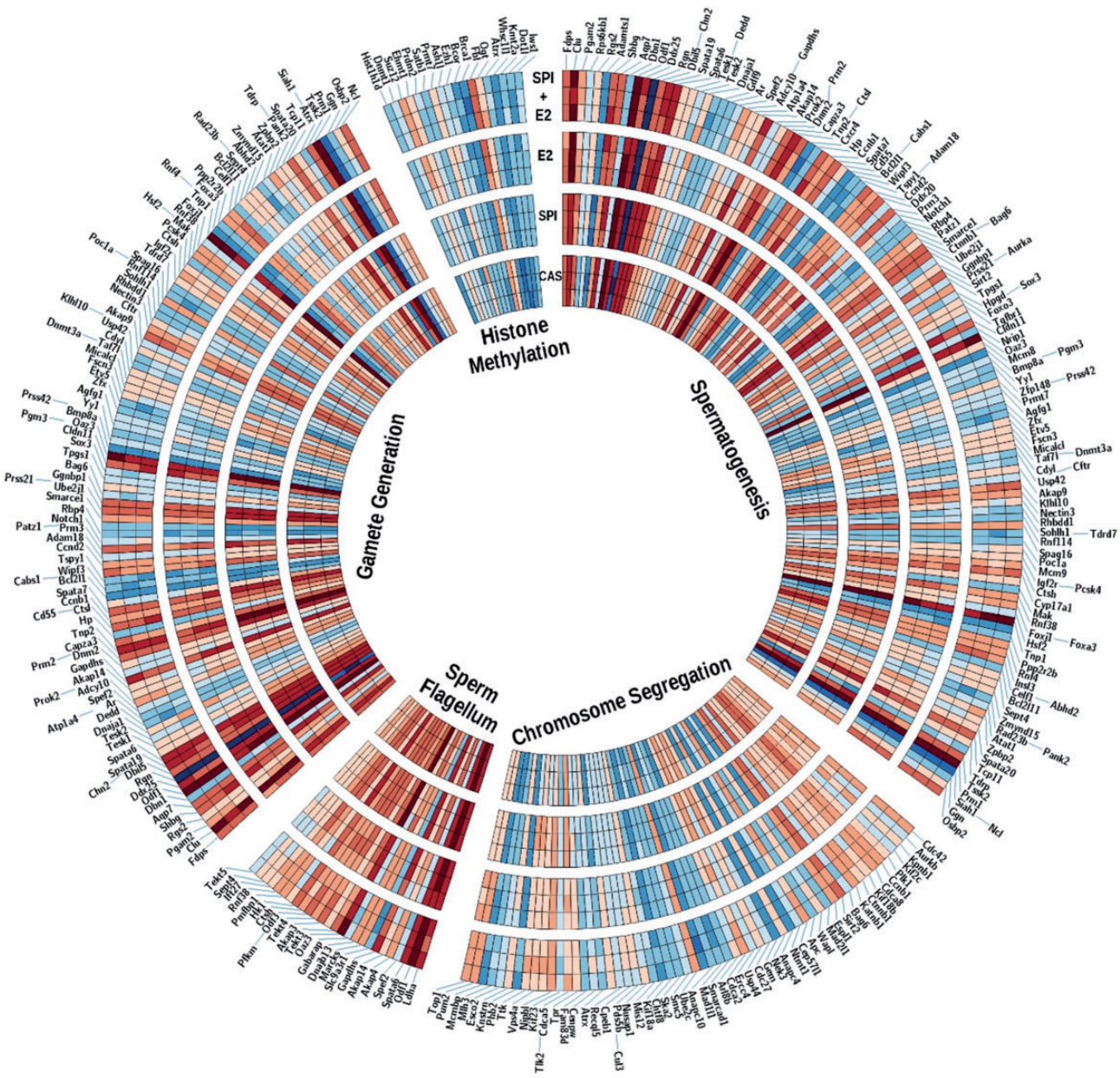

Figure 2. RNA mRNA heat maps of testicular genes changed by >1.5-fold in prepubertal male rats by treatment with E2, feeding SPI, or SPI +E2 treatment relative to rats fed casein. Genes were separated in circular bands Outer band: E2 + SPI; middle band: SPI; inner band E2. Detailed gene expression lists can be found online at the National Center for Biotechnology Information (http:/www.ncbi.nlm.nih.gov/geo/) under GEO accession no. GSE94392. (A color version of this figure is available in the online journal.)

feeding may not share the same properties as an individual component, (2) SPI isoflavones undergo an extensive first pass conjugation process in the GI tract, resulting in very low nM concentrations of bioactive phytoestrogens reaching the tissue, and (3) endogenous estrogens have a higher affinity for ER receptors than isoflavones resulting in significant competition for the ER receptors even at low levels found in the pre-pubertal male.[1,2,21–25]

To directly compare the estrogenic actions of exogenous E2 supplementation with that of feeding SPI on the reproductive axis, male rats were treated from weaning on PND 21 until the beginning of puberty on PND 34. As expected, E2 treatment resulted in reduced body weight, testis and secondary sex organ weight, and dramatic reductions in the serum concentrations of the androgens testosterone and DHT. Array analysis revealed that E2 treatment also significantly affected the expression of 1639 testicular genes >1.5-fold of which nearly 2/3 were down-regulated ($P < 0.05$). This included many genes associated with reproductive function including mRNAs encoding genes in the steroidogenic pathway.

Sex and adrenal steroids are synthesized from cholesterol via stimulation of cholesterol transport within the mitochondria by the sterol acute regulatory protein (StAR) and the formation of pregnenolone by the mitochondrial cytochrome P450 11a1 (Cyp11a1).[52] In the testis, conversion of pregnenolone to progesterone occurs via 3β hydroxysteroid dehydrogenase (3β-Hsd) and Cyp17a1 catalyzes conversion of progesterone to androstenedione.[53]

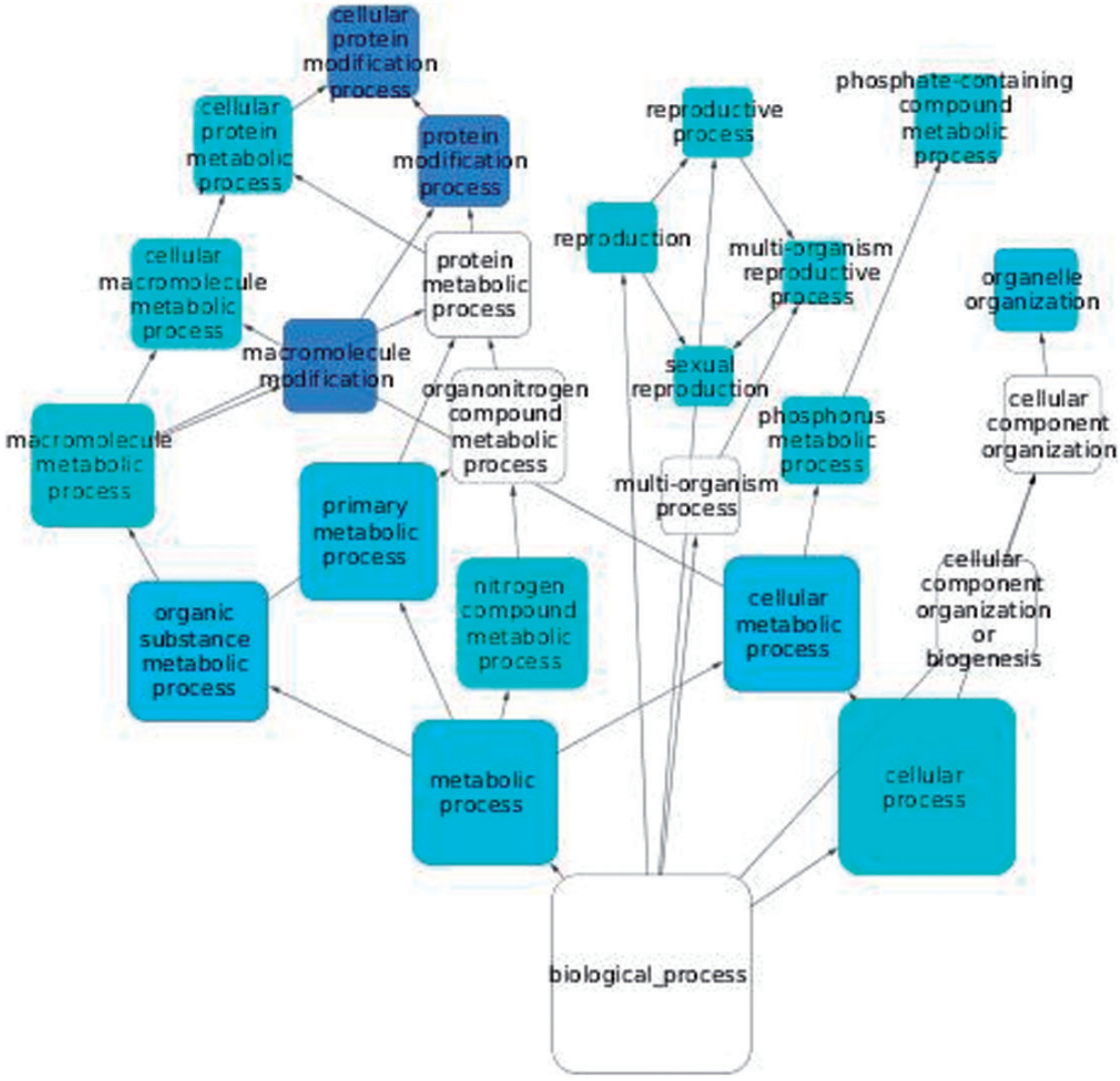

Figure 3. Functional analysis of gene expression ontology in the prepubertal rat testes for E2 treatment vs. casein control. Adjusted *P*-values range from 10^{-8} (dark blue) to 10^{-4} (light blue) based on the core ontology (Biological Process) from the gene ontology consortium. Size of the node is relative to the number of genes on the given biological process. (A color version of this figure is available in the online journal.)

Androstenedione is then converted to the androgen testosterone by 17β-Hsd3 and testosterone is further metabolized into DHT by steroid 5α-reductase 1 (SRD5a1).[54] Androgen production occurs mainly in Leydig cells and is under regulation by the pituitary gonadotropin luteinizing hormone (LH).[55] Alternately, androstenedione and testosterone can be converted to the estrogens estrone (E1) and E2 by the actions of aromatase (Cyp19). 17β-Hsd1 converts E1 to E2 and 17β-Hsd2 converts E2 to E1.[53] In the major adrenal pathway, pregnenolone is conversed to dehydroepiandrosterone (DHEA) by Cyp17a1 which is then sulfated to form the major circulating adrenal steroid DHEA-S.[56] On the other side of the equation, steroids are degraded primarily in the liver via hydroxylation by cytochrome P450 enzymes and by sulfate and glucuronide conjugation.[57]

Although we present data that both Star and Cyp17a1 mRNA and protein were suppressed in the testis by E2 treatment, consistent with reduced serum testosterone and DHT, we observed no effect on serum pregnenolone, progesterone, or androstenedione concentrations. This suggests that testicular expression of these enzymes is not rate limiting in the synthesis of circulating androgens at this age. These data are consistent with a rate limiting role for 17β-Hsd3 activity. 17β-Hsd3 is required for testicular conversion of androstenedione to testosterone and adrenal conversion of DHEA to androstenedione. Consistent with this possibility, E2 treatment also significantly increased serum DHEAS and testicular 17β-Hsd3 mRNA was suppressed by E2 treatment in our array data. We did observe significant E2-associated increases in serum E1 in both

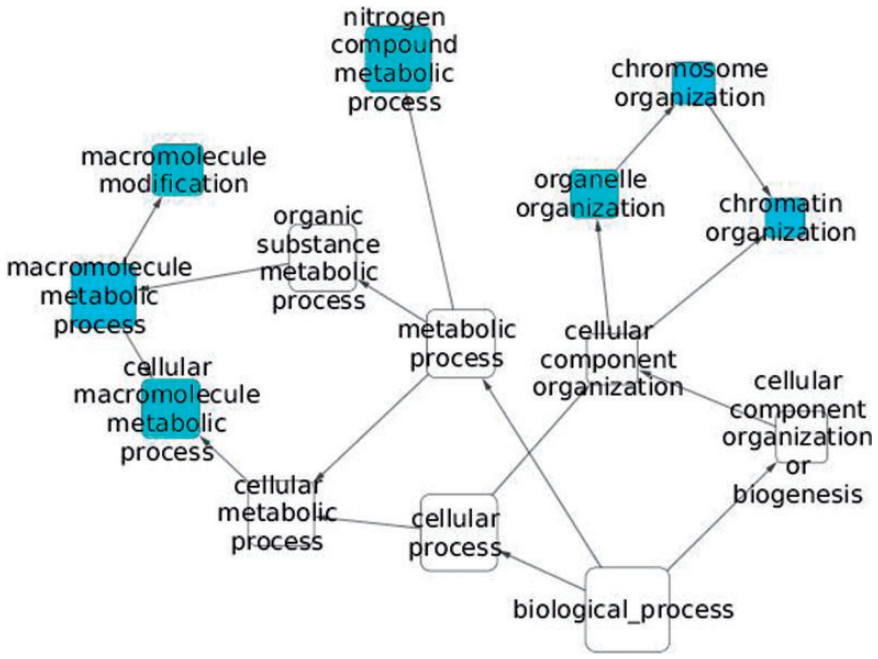

Figure 4. Functional analysis of gene expression ontology in the prepubertal rat testes for SPI feeding vs. casein control. Adjusted *P*-values range from 10^{-8} (dark blue) to 10^{-4} (light blue) based on the core ontology (biological process) from the gene ontology consortium. Size of the node is relative to the number of genes on the given biological process. (A color version of this figure is available in the online journal.)

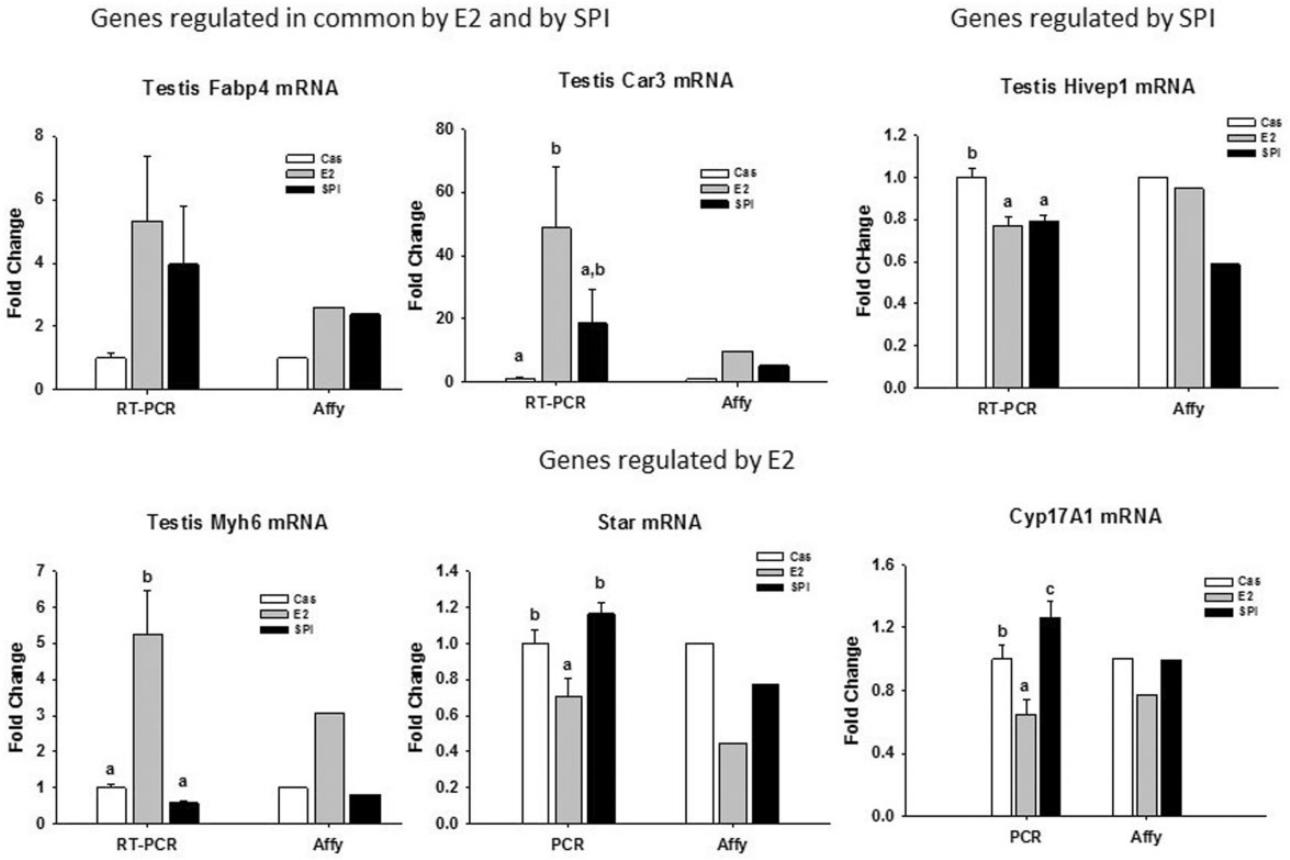

Figure 5. Real-time RT-PCR confirmation of gene regulation in prepubertal rat testis by E2 treatment or SPI feeding. Comparison of real time data (RT-PCR) with Affymetrix array values (Affy). Real-time RT-PCR data are mean ± SEM. Means with different letters are significantly different $P < 0.05$ by one-way ANOVA, b > a.

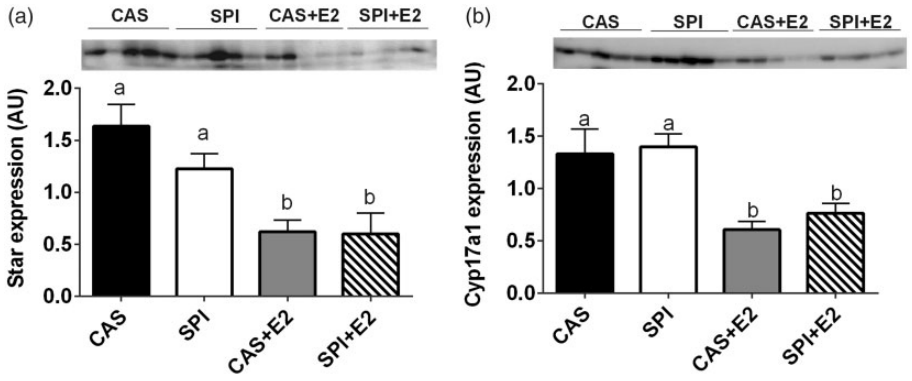

Figure 6. Left hand panel: Western blot analysis of Star protein in rat testes exposed to CAS, SPI, CAS + E2, and SPI + E2 diets. Statistical significance was determined by two-way ANOVA followed by Student–Newman–Keuls *post hoc* analysis; E2-treatment ($P < 0.001$), but not diet ($P = 0.225$) had a significant effect on Star protein expression; interaction ($P = 0.276$). Right hand panel: Western blot analysis of Cyp17a1 expression in rat testes exposed to CAS, SPI, CAS + E2, and SPI + E2 diets. Statistical significance was determined by two-way ANOVA followed by Student–Newman–Keuls *post hoc* analysis; E2-treatment ($P < 0.001$), but not diet ($P = 0.457$) had a significant effect on Star protein expression; interaction ($P = 0.758$). Groups with different letter subscripts are significantly different from each other.

CAS + E2 and SPI + E2 groups compared to the CAS group. Increased serum E1 after E2 treatment may simply reflect the higher levels of E2 substrate for 17β-Hsd2. Replacement of CAS in the diet by SPI had no effect on serum testosterone or DHT but did result in significant reductions in serum androstenedione and increases in DHEAS in both the SPI group and in the SPI + E2 group relative to the CAS group. It is possible this reflects selective inhibition of adrenal 17β-Hsd3 by components of SPI. Feeding SPI compared to CAS also resulted in significant increases in serum E1 but not E2. This could be associated with effects of SPI components to increase aromatase catalyzed conversion of androstenedione to E1. Additional work is required to assess the possible effects of SPI feeding on these aspects of steroid metabolism.

Isocaloric replacement of CAS with SPI in the diets resulted in a significant reduction in body weight in either the presence or absence of additional E2 treatment. Such reductions in body weight and improvement in body composition as the result of reduced adiposity are a consistent feature of feeding studies with soy products and SPI in multiple species and has been ascribed variously to effects on the growth-hormone-IGF-1 axis, increases in fatty acid metabolism via activation of PPARα or effects on adipogenesis via the ERβ pathway.[58–60] However, in contrast to E2 treatment, feeding diets where SPI was used to replace CAS as the protein source had no effects on the weight of male reproductive organs or on serum testosterone or DHT concentrations. We observed no evidence of additive effects of SPI + E2 on these endpoints. These data are in agreement with previous studies in our laboratory of male reproductive function and endocrinology in rats fed SPI throughout gestation and development.[26] In humans, there was no effect of postnatal soy formula feeding on testis and prostate growth in children at one and five years of age.[50,51] Array analysis revealed a 10% overlap between E2 and SPI-regulated testicular genes and almost all of the SPI-regulated genes overlapped with E2 up-regulated pathways associated with macromolecule modification as opposed to overlapping with E2 down-regulated pathways associated with androgen synthesis and reproductive development. These data may reflect competition between isoflavones and endogenous estrogens for ER-signaling pathways or differences between E2 and soy phytochemicals in activation of ER signaling via ERα and ERβ by selectively recruiting specific co-activators and co-repressors, similar to selective estrogen receptor modulator (SERM) responses observed in previous studies.[21–23] SPI feeding produced similar effects to E2 in bone of pre-pubertal rats; however, overlap between E2 and SPI-signaling networks was observed to be much greater in bone suggesting that interactions of SPI with ER-signaling pathways may be organ-dependent.[25]

Our findings are at variance with several developmental soy studies reporting suppressed steroidogenesis, decreased testosterone concentrations, and increased Leydig cell proliferation in rat offspring exposed to diets made with soybeans during perinatal period.[61–63] However, there are significant differences in experimental design between this and the current study. Napier *et al.* used Long-Evans rats compared to Sprague Dawley rats in the current experiment; exposure was to a whole soybean diet which may have a significantly different phytochemical composition compared to the current SPI-based AIN-93G diet; and the exposure route and window were different. Napier *et al.* exposed rat pups via the dam during PND 1–21, whereas the current study utilized direct dietary exposure from PND 21–33. Moreover, the serum concentrations of isoflavone aglycones differ considerably between the two studies. In the current study, total serum aglycone values were 60–100 nM compared to 370 nM in Napier *et al.* Since the rats were sacrificed at 0800 h in the morning and the half-life of isoflavones is 3–8 h,[57,64] peak concentrations of circulating isoflavone aglycones after consumption of food during the dark cycle may have been even more divergent.

Sharpe and his co-workers[47,48] have reported reduced serum testosterone and increased Leydig cell numbers in marmoset monkeys following postnatal feeding of soy infant formula although no lasting effects were observed

with respect to fertility, testis size, or Leydig cell numbers at adulthood, indicating altered reproductive health However, these data also differ from the weak estrogenic effects reported on the testis after developmental exposure to pure genistein.[65] In contrast, chronic feeding studies with SPI in adult male cynomolgus macaque monkeys had no effect on testis weight, morphology, serum testosterone or E2 concentrations or sperm counts.[66] It is possible that the variable responses in monkeys are due to differences in soy preparations (SPI vs. whole soybean vs. soy formula) and species differences in isoflavone metabolism. The major serum isoflavone in the serum of monkeys fed soy is equol, which makes up only a small portion of the free isoflavones found in serum from the current rat study and is not made by human infants prior to weaning.[27]

Although there is no literature suggesting sex differences in isoflavones pharmacokinetics in the rat,[64] the present data demonstrate significant differences in serum daidzein conjugates and in free genistein and daidzein isoflavone concentrations after E2 treatment of pre-pubertal male rats consuming SPI. The increase in total daidzein may be the result of E2 actions on daidzein metabolism in the GI by gut bacteria, since although not significant there was a corresponding increase in serum total equol values and daidzein is converted to equol by the microbiota. Increases in isoflavones aglycones may represent E2-asociated decreases in glucuronide and sulfur conjugation of the aclycones during absorption from the GI. Therefore, the difference in free isoflavone concentration may contribute to the altered gene expression profiles observed in the SPI + E2 group relative to the CAS + E2 group or SPI group. The current study has a number of limitations if the findings are to be applied to assessment of the safety of soy infant formula feeding to male infants. Since it is not possible to formula feed neonatal rats, the SPI diet is solid rather than liquid and the exposure window is later than in the testis of a human infant exposed to soy formula. However, there is no quiescent period in rat testicular development as occurs in humans and the later juvenile period in the rat is considered a sensitive time point for assessment of testicular responses to endocrine disruptors.[4] The first wave of spermatocyte development begins on PND 18 and is sustained by the production of DHT which peaks between PND 20 and 40 and final maturation of Leydig cells and of the hypothalamo-pituitary-gonadal axis occurs around PND 25–30.[54,67] The comparison of SPI feeding is with a control AIN-93G diet where the protein source is casein. AIN-93G diets are the standard control diets used in nutritional research;[22,68] casein is the principal protein in cow's milk formula diets. It has been suggested that SPI diets stripped of phytochemicals might be a more appropriate control since casein may have its own biological effects.[68] However, the processing required to remove phytochemicals also alters the protein/peptide composition of SPI and may also change its biological properties. An additional limitation is lack of comparison to a group fed purified isoflavones. However, matching such a group to SPI-fed animals would be also be difficult. Even adding an isoflavone mixture which mimics the composition found in SPI to an AIN-93G CAS diet might not produce serum aglycone values similar to those seen after feeding SPI. Differences in protein matrix and gut microbiota could result in significant differences in isoflavone metabolite profiles and pharmacokinetics.

In conclusion, our data do not support the contention that SPI or soy formula has estrogenic actions or indications of reproductive toxicity, including reduced testicular or prostate growth or androgen production, in early male development. SERM-like effects are observed resulting in changes in a small sub-set of testicular genes linked to macromolecule modification. However, it is unclear what, if any, effects these changes in gene expression have on testicular development or if such changes occur in human infants given differences in soy formulation, developmental window, and isoflavone metabolism. Additional studies of morphology, intracrinology, and estrogenic pathways in the testis, adrenals, and secondary sex organs are required to further assess the potential for reproductive toxicity of soy infant formula using more appropriate models of male neonatal development such as the piglet where infant formulas can be fed directly during the appropriate developmental window and where isoflavone metabolism is more similar to that in human infants.

Authors' contributions: All authors reviewed the manuscript and performed data analysis. MJR, KS, RS, TMB participated in research design. NS, MB, RS, and RS conducted experiments. HGA conducted statistical analysis. MJR and KEM wrote or contributed to the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

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