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Long-term effects of dietary isoflavones on uterine gene expression profiles

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ABSTRACT

Isoflavones (ISOs) are bioactive food ingredients of the traditional East Asian diet and currently discussed as alternatives to classical hormone replacement therapies and for reducing the prevalence of hormone-dependent cancers. Although there are many studies on ISOs, not much is known about their long-term effects.

Therefore, we performed an animal experiment analyzing the effects of three different diets: a phytoestrogen-free diet, a diet supplemented with genistein (700 μg/g diet) and an ISO-high diet (232 μg daidzein and 240 μg genistein/g) at two distinct time points, juvenile (21 days) and adult (97 days). Exposure started prior to mating of the parents and throughout the life of the offspring.

We observed a stronger increase of uterine wet weights in juvenile offspring with genistein exposure (1018 ± 350 mg/kg BW) than with ISO-high diet (497 ± 133 mg/kg BW). Whereas the expression of proliferation related genes (*PCNA*; *Ki67*; *IGF-1*; *IGF-1R*), analyzed by real-time-qPCR and Western blot, were significantly down-regulated in juvenile animals exposed to genistein. Additionally, genistein exposure led to estrogenic responses, observed upon increase of complement C3 and decrease of estrogen receptors gene expressions, while the exposure to ISO-high diet did not show these effects.

In conclusion, both the time point on which phytoestrogen exposure starts together with the composition of the ingested phytoestrogen containing diet are of great importance for the biological response of the offspring.

1. Introduction

Recent years have seen a growing research focus on bioactive plant-derived compounds exerting endocrine effects. In particular for phytoestrogens (PEs) there are claims by companies that they are potential alternatives for the classical hormone replacement therapy. As a consequence they are already widely used for the treatment of post-menopausal complaints including hot flushes, sleep and mood disturbances.

Because PEs share structural similarities to the mammalian steroid hormone 17β-estradiol (E_2) as well as to the promiscuity of the associated receptors, PEs are able to bind to both estrogen receptor (ER) subtypes and subsequently initiate estrogen-dependent transcription [1].

Besides inducing mainly weak estrogenic or anti-estrogenic responses *in vivo*, PEs exhibit several other (non)-hormonal effects. These include for instance the reduction of free circulating endogenous hormones by increasing the concentration of sex hormone binding globulins [2]. In addition, they modulate the activities of steroidogenic enzymes, act as anti-oxidants at relatively high concentrations and can inhibit cellular signalling transduction [3–7].

Since the ligand binding domains of the ER subtypes (ERα and ERβ) show only a 55% homology and the distribution of the ER subtypes is tissue-dependent, it is not surprising that these features result in multiple miscellaneous physiological effects [8,9]. For the ligand binding pocket of both receptors, ample space around the ligand could be shown, enabling a variety of molecules to bind into this binding pocket, e.g., PEs. These polyphenolic non-steroidal dietary estrogen-like substances, found in more than 300 different plants [10], are mainly divided into isoflavones (ISOs), flavanones, coumestanes and lignans [1,11–13]. The most intensively investigated representatives are the ISOs, namely genistein (GEN) and daidzein (DAI), which are found as beta-glycosides almost exclusively in legumes. Their precursors (i.e., genistin and daidzin, respectively) are converted in the mammalian gut via glucosidases and/or bacterial microflora to the respective active aglycones, or further downstream to metabolites like equol, which subsequently act as potent activators of different biological effects [6,9,14,15].

Soybeans and soy products such as tofu are a rich source for ISOs. For instance, soybeans contain approximately 0.3–1.2 mg GEN and 0.4–1.1 mg DAI/g fresh weight [16]. Considering these values, in combination with the fact that soy is a basic component

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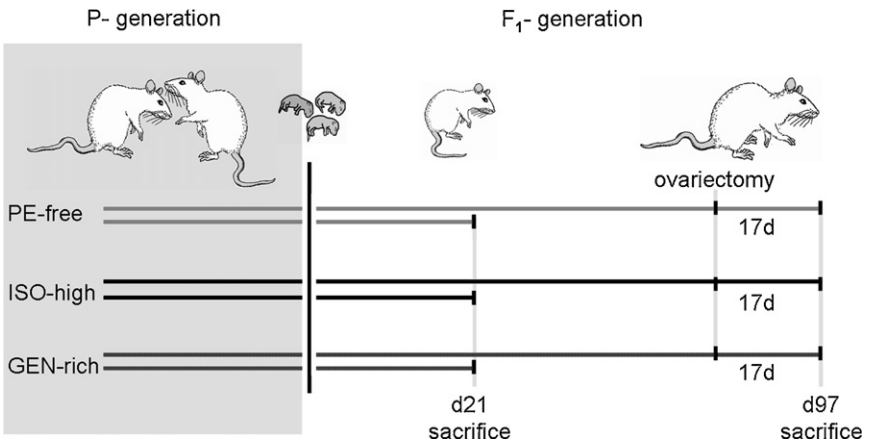

Fig. 1. Experimental design.

in traditional East Asian diet, warrants the assumption that the lower incidences of hormone-dependent cancers observed in this region are associated with the high PE intake. A small number of epidemiological, clinical and experimental studies were carried out to investigate this negative correlation between the intake of dietary PEs, especially ISOs, and the prevalence of endometrial or breast cancer [17–19]. Unfortunately, some clinical studies show controversial data. D'Anna and co-workers observed in a 1-year randomized, double-blind, placebo-controlled study that a dietary supplementation of postmenopausal women with 54 mg ISOs/day had no impact on the mean endometrial thickness [20]. Whereas Unfer and co-workers showed in a randomized, double-blind, placebo-controlled study that a treatment with daily 150 mg ISOs may lead to endometrial hyperplasia after 5 years [21]. Therefore, further studies are needed to determine whether the effects of dietary ISOs in humans are beneficial, indifferent or adverse.

Moreover, a recent set of data supports the theory that the potential chemoprotective property of PEs is dependent on the duration of PE exposure (e.g., lifelong exposure from prenatal through neonatal into adolescent and adult ages) [22]. In accordance, a retrospective study has shown that Chinese breast cancer patients in average had a lower soy food intake during adolescence as compared to age-matched control individuals. This may indirectly indicate that early PE exposure leads to beneficial effects during adulthood [23].

In the present study, we mimic the high ISO consumption of East Asian diets to verify epidemiological observations in an *in vivo* experimental setting. We addressed the question of whether a lifelong exposure to PEs (started during the embryonic development, neonatal, pre-pubertal up to adulthood stages) could result in both general modifications of physiological homeostasis and different gene regulatory patterns in the uterus. Therefore, female Wistar rats were fed different diets: The control group received a phytoestrogen-free diet (PE-free) mimicking the PE content of a typical Western diet. The second diet contained high levels of isoflavones (ISO-high), with equal amounts of GEN and DAI. This diet simulates the PE exposure of the traditional East Asian diet. The third group received a GEN-supplemented diet (GEN-rich) representing the Western diet supplemented with GEN. We compared the impact of the three different diets by measuring the uterine wet weights (UWW) at two developmental stages: pre-pubertal and adult. Additionally, blood plasma levels of GEN and DAI as well as its metabolite equol were measured. Finally, the expression of estrogen-dependent uterine target genes such as relevant steroid receptors (estrogen receptors α and β, progesterone receptor), distinguished estrogenic marker genes in the rat uterus (clusterin and complement C3), proliferation associated genes, like *PCNA*, Ki67 as well as the insulin-like growth factor 1 and its receptor were quantified by qPCR and Western blot analysis.

2. Materials and methods

2.1. Animals and diets

The experimental design is shown in Fig. 1. All animal handling and experimental conditions were carried out according to the Institutional Animal Care and Use Committee guidelines as regulated by the German federal law governing animal welfare. Animals were housed under controlled conditions of temperature ($20 \pm 1\,^{\circ}C$; relative humidity 50–80%), illumination (12 h light/12 h dark) and had *ad libitum* access to the diets and water. The parental generation consisted of twenty-three male and thirty-eight female Wistar rats of 10 weeks of age (225–250 g) obtained by Janvier, Le Genest St Isle, France. The breeding pairs were randomly split into three feeding groups. The first group received a phytoestrogen-free diet (PE-free) (Ssniff SM R/M-H, 10 mm, phytoestrogen-free) obtained from Ssniff (Soest, Germany). The second group obtained an isoflavone-high diet (ISO-high) produced from dehulled soybean meal, containing 232 ± 10 μg DAI and 240 ± 36 μg GEN/g (Harlan Teklad 8604 rodent diet) [24]. This diet was purchased from Harlan Teklad (Borchen, Germany). The third group received a genistein-rich diet (GEN-rich), containing 700 μg/g GEN (Ssniff SM R/M-H, 10 mm, phytoestrogen-free added by 700 μg/g GEN), obtained from Ssniff (Soest, Germany). The GEN-rich diet was custom-made with GEN (4′,5,7-trihydroxyisoflavone) provided by LC Laboratories (Woburn, USA). Gross and metabolizable energies of all diets were balanced and are shown in Table 1.

Pregnant females were kept under the established dietary regimen throughout gestation and lactation period. Only female offspring was further investigated in the following experimental procedures. At day 21 post-partum six females per group were sacrificed by CO_2 inhalation after light anaesthesia with O_2/CO_2 (1:1) inhalation. Uteri were collected and weighed after removal of associated fat, and snap frozen in liquid nitrogen for RNA and protein preparation. The remaining female offspring ($n = 6$/group) was maintained under the established dietary regimen until adulthood. 80 days post-partum, these animals were ovariectomized (OVX) to reduce the endogenous hormonal background. The resulting minimal hormone levels are important to visualize the comparatively weak impact of the dietary ISOs. After 17 days of endogenous hor-

Table 1
Phytoestrogen (PE) contents, gross energies (GE) and metabolizable energies (ME) of used diets. Estimated daily PE intake of daidzein (DAI) and/or genistein (GEN) at the investigated time points.

	Diet					
	PE-free		ISO-high		GEN-rich	
Energies [MJ/kg diet]	16.5^{GE}	12.8^{ME}	16.4^{GE}	13.0^{ME}	16.5^{GE}	12.8^{ME}
PE content of diet [per gram]	<10 μg DAI and GEN		232 ± 10 μg DAI 240 ± 36 μg GEN		~700 μg GEN	
PE intake (juvenile) [mg/kg BW and day]	<1		49 (DAI + GEN)		73	
PE intake (adult) [mg/kg BW and day]	<1.3		63 (DAI + GEN)		93	

monal decline these animals were sacrificed. The uterine collection followed the same protocol as described above.

2.2. RNA preparation, reverse transcription and real-time qPCR

The total RNA was extracted from the uteri according to the standard TRIzol® method described by Chomczynski and Sacchi [25] using the peqGOLD TriFast™ protocol (PeqLab, Erlangen, Germany). RNAs from the same treatment group were pooled and the DNA contaminations were enzymatically eliminated by digestion (Desoxyribonuclease 1, Roche Diagnostic GmbH, Mannheim, Germany). Success of DNA digestion was verified by PCR. M-MVL-Reverse Transcriptase (Promega, Madison, USA) and Oligo $(dT)_{12–18}$-primers were used for the first-strand cDNA synthesis.

Resulting cDNA was amplified by quantitative real-time PCR (qPCR), performed with Platinum® *Taq* DNA polymerase (Invitrogen, Karlsruhe, Germany) using the iCycler Thermal Cycler with iO real-time Detection System (BIO-Rad Laboratories GmbH, München, Germany). SybrGreen® I (Sigma–Aldrich, Taufkirchen, Germany) was used as detection dye. The expression of all genes was normalized against the housekeeping gene *cytochrome-c-oxidase subunit 1A* (*1A*). Table 2 shows the primer pairs used for the qPCR experiments. All genes were analyzed at least three times as triplicates using independently produced cDNAs of the same sample. PCR was performed with 50 μl aliquots in a 96-well plate while the program consisted of a first denaturation step at 95 °C for 3 min followed by 50 cycles of 10 s at 95 °C, 10 s at 60 °C and 20 s at 72 °C. The fluorescence was quantified during the 72 °C elongation step and the product formation was confirmed by melting curve analysis (60–96 °C). Relative rates of gene expression were calculated using the $\Delta\Delta C_T$ method [26]. Animals of the PE-free diet group were used as control and their expression data was defined as 1 (black line in Figs. 3–5A). All gene expression profiles were normalized to control.

Table 2
Primer pair sequences and amplicon sizes. *1A*: *cytochrome-c-oxidase subunit 1A*, *ERα*: estrogen receptor alpha, *ERβ*: estrogen receptor beta, *PR*: progesterone receptor, *Clu*: clusterin, *C3*: complement C3, *PCNA*: proliferating cell nuclear antigen, *Ki67*: antigen identified by monoclonal antibody Ki67, *IGF-1*: insulin-like growth factor 1, *IGF-1R*: insulin-like growth factor 1 receptor.

Primer	Direction	Sequences	Amplicon size [bp]
1A	fwd rev	5′-tga gca gga ata gta ggg aca gc-3′ 5′-gag tag aaa tga tgg agg aag ca-3′	260
ERα	fwd rev	5′-gga agc aca agc gtc aga gag at-3′ 5′-aga cca gac caa tca tca gga t-3′	382
ERβ	fwd rev	5′-cta cag aga gat ggt caa aag tgg a-3′ 5′-ggg caa gga gac aga aag taa gt-3′	215
PR	fwd rev	5′-ccc aga cga aaa gac aca aaa t-3′ 5′-cca aag aga cac caa gaa gtg at-3′	221
Clu	fwd rev	5′-ccc tcc agt cca aga tgc tca aca c-3′ 5′-cca tgc ggc ttt tcc tgc ggt att c-3′	302
C3	fwd rev	5′-aca gcc ttc ccg gga gca tca aca-3′ 5′-agc gca cca cag gag gca cag agt c-3′	275
PCNA	fwd rev	5′-gag caa ctt gga atc cca gaa cag g-3′ 5′-cca agc tcc cca ctc gca gaa aac t-3′	157
Ki67	fwd rev	5′-aac cag gac ttt gtg ctc tgt aa-3′ 5′-ctc ttt tgg ctt cca ttt ctt c-3′	208
IGF-1	fwd rev	5′-ctg ctt gct cac ctt tac cag-3′ 5′-tac atc tcc agc ctc ctc aga-3′	212
IGF-1R	fwd rev	5′-gtg gag gag gtg aca gaa aat c-3′ 5′-caa aga tgg agt tgt gaa gga a-3′	156

2.3. Western blot

Frozen tissue was pulverized in liquid nitrogen and homogenized in a mixture (1:1) of preparation buffer (240 mmol l^{-1} saccharose; 20 mmol l^{-1} PIPES; 10 mmol l^{-1} EDTA; 50 mmol l^{-1} NaH_2PO_4; 1 mmol l^{-1} PMSF in 2-propanol; pH 7.4) and freezing buffer (400 mmol l^{-1} saccharose; 5 mmol l^{-1} PIPES; 5 mmol l^{-1} Tris; 10 mmol l^{-1} EDTA; 50 mmol l^{-1} NaH_2PO_4; pH 7.2). Protein concentration was determined using bicinchoninic acid (VWR, Darmstadt, Germany). Western blot was performed with three independent investigations of protein samples. Equal amounts of protein samples (30 μg) were loaded onto a 8.5% SDS-polyacrylamide gel, electrophoresed and transferred to 0.45 μm PVDF membranes (Millipore, MA, USA). The PVDF membranes were blocked with 5% skim milk powder and incubated with specific antibodies for proliferating cell nuclear antigen (PCNA) (1:1000, Dako Cytomation, Glostrup, Denmark) and actin (1:4000, Sigma, Saint Louis, MO, USA) at room temperature for 1 h, followed by incubations with goat-anti-mouse horseradish-peroxidase (HRPO)-conjugated secondary antibody (1:10,000, Dianova, Hamburg, Germany) or goat-anti-rabbit HRPO conjugated secondary antibody (1:30,000, Dianova, Hamburg, Germany), respectively, at room temperature for 1 h. The Western blots were developed with a chemiluminescence ECL Plus Western blotting detection system (GE Healthcare, Buckinghamshire, UK) and exposed to Amersham Hyperfilm ECL (GE Healthcare, Buckinghamshire, UK) at room temperature. The protein bands were quantified by densitometry using the LabWorks 4.6 software (UVP Ltd, Cambridge, UK). The ratio of the reference protein (actin) to the target protein (PCNA) was calculated to account for inhomogeneous protein loading. Animals of the PE-free diet group were used as control and their expression data was defined as 1 (black line in Fig. 5B).

2.4. Statistical analysis

The data of the uterine wet weights are presented as median ± standard deviation whereas the $2^{-\Delta\Delta C_T}$ values of the real-time qPCR are presented as arithmetic mean ± standard deviation using MS-Excel. Statistical analysis of the uterine wet weight data included ANOVA followed by Bonferroni post hoc test to determine significant differences ($p \leq 0.05$). The standard Student's *t*-test was

used to detect statistically significant differences of gene expression profiles ($p \leq 0.05$).

3. Results

3.1. Dietary PE intake and plasma levels

The effects of the three different animal diets on uterine physiology and molecular parameters were analyzed and compared at two different developmental stages (juvenile and adult). The PE-free diet contained less than 10 μg DAI and GEN/g diet. The ISO-high diet contained 232 ± 10 μg DAI and 240 ± 36 μg GEN/g, and the GEN-rich diet was composed of the PE-free diet supplemented with 700 μg GEN/g. Values of daily PE intake were estimated according to the daily food intake of approximately 7.5 g per animal (average body weight 72 g) in the case of juvenile animals. Regarding adult rats with an average body weight (BW) of 150 g, the average of daily food intake was 18.4 g.

Table 1 summarizes the experimental PE exposures. The control animals fed with the PE-free diet had negligible exposure to dietary PEs. Juvenile animals ingested 49 and 73 mg ISOs/kg BW daily in the ISO-high and GEN-rich group, respectively. During adulthood PE-levels increased 30% to a daily intake of 63 and 93 mg/kg BW in the ISO-high and GEN-rich group, respectively. For juvenile animals, the plasma levels of GEN, DAI and equol were measured by Hertrampf et al. (details in Hertrampf et al. 2009, submitted for publication). PEs and metabolite levels in the blood plasma of the animals fed the PE-free diet were close to detection limit (<12 ng GEN, <6 ng DAI and <3 ng equol/ml plasma). Animals of the ISO-high diet group showed high PE-levels (468 ± 159 ng GEN, 338 ± 96 ng DAI, 821 ± 174 ng equol/ml plasma) and animals of the GEN-rich diet group had PE-levels of 1038 ± 946 ng GEN, <6 ng DAI, <3 ng equol/ml plasma.

3.2. Uterine wet weight (UWW)

The uterus is a major target tissue for endogenous estrogens as well as xenoestrogens. Therefore, data derived from uterine physiology and uterine specific gene regulations are used to evaluate estrogenic potentials of substances. Even though no differences of BW of age-matched animals of the dietary groups were detectable, neither for the juvenile (PE-free: 79.6 ± 12.0 g; ISO-high: 68.1 ± 15.0 g and GEN-rich: 68.6 ± 6.8 g) nor for the adult animals (PE-free: 295.6 ± 18.9 g; ISO-high: 298.6 ± 25.6 g and GEN-rich: 294.3 ± 27.8), their UWWs were normalized against the BW to obtain a more precise evaluation and a better comparability.

In the PE-free diet control group, the UWWs of the juvenile (508 ± 177 mg/kg BW) and adult rats (457 ± 61 mg/kg BW) were on the same level. In juvenile animals, there was no significant difference of UWWs between the ISO-high diet (497 ± 133 mg/kg BW) and the PE-free group. However, a significant increase of the UWW was detectable in the GEN-rich group (1018 ± 350 mg/kg BW) compared to the PE-free control. In the adult OVX animals, both diets the ISO-high and GEN-rich led to weak but significant increases of UWWs (673 ± 67 mg/kg BW and 623 ± 40 mg/kg BW, respectively) (Fig. 2).

3.3. Gene and protein expression

In addition, PE exposure led to changes in the expression pattern of estrogen-responsive genes in the uterus. The expression levels of the following genes were investigated by qPCR. We investigated relevant steroid receptors like both estrogen receptor subtypes (*ERα*/*ERβ*) and the progesterone receptor (*PR*). Additionally, we focussed on distinguished estrogenic response genes, i.e., clusterin (*Clu*) and complement C3 (*C3*) [27]. And finally, we addressed genes

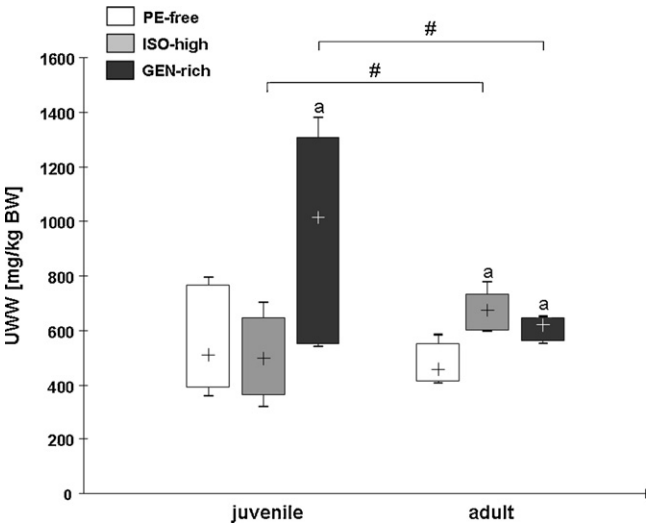

Fig. 2. Uterine wet weights (UWW) of juvenile and adult Wistar rats after long-term exposure to the respective dietary isoflavones. The box plot shows the 10 and 90% percentiles (box), the median (+) as well as the minimum and maximum values. Wet weights differ significantly from the respective PE-free control group: $^{a}p < 0.05$ calculated by ANOVA followed by post hoc Bonferroni test. Wet weights differ significantly between juvenile and adult animals within the same diet group: $^{\#}p < 0.05$ calculated with standard Student's *t*-test.

associated with proliferation in the uterus, the two proliferation markers namely the proliferating cell nuclear antigen (*PCNA*) and the antigen identified by monoclonal antibody Ki67 (*Ki67*) as well as the paracrine mediators of estrogenic proliferation insulin-like growth factor 1 (*IGF-1*) and its receptor (*IGF-1R*).

The mRNA expression of both ER subtypes was not affected by the ISO-high diet in juvenile and adult rat uteri (Table 3). In contrast, the juvenile animals of the GEN-rich diet showed a highly significant down-regulation (ERα: 0.20 ± 0.12 fold and ERβ: 0.29 ± 0.02 fold) of both ER subtypes. For *ERα*, this lower expression rate compared to the control group could also be detected in the adult animals, whereas in these animals *ERβ* expression was apparently abrogated. In juvenile animals the ISO-high diet led to an up-regulation of *PR* expression (2.03 ± 0.29 fold), whereas a significant down-regulation (0.27 ± 0.13 fold) was noticed in the GEN-rich diet group. In adult animals the *PR* mRNA levels were converse. We detected a significant down-regulation (0.48 ± 0.08 fold) in animals receiving the ISO-high diet whereas the GEN-rich diet had no effect (Table 3).

With regard on well-known estrogenic marker genes in the uterus, neither the ISO-high nor the GEN-rich diet significantly affected the regulation of *Clu* at any observed time point.

Table 3
Impact of long-term dietary exposure to phytoestrogens on mRNA levels of estrogen sensitive genes. The table shows the regulation patterns of the pre-pubertal and adult animals. Displayed are the changes of the mRNA expression levels of the respective genes compared to the PE-free diet. Values less than one represent a down-regulation whereas values greater than one represent an up-regulation. Expression differs significantly from the PE-free control group: $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. *ERα*: estrogen receptor alpha, *ERβ*: estrogen receptor beta, *PR*: progesterone receptor, *Clu*: clusterin, *IGF-1*: insulin-like growth factor 1, *IGF-1R*: insulin-like growth factor 1 receptor.

Genes	ISO-high		GEN-rich	
	Juvenile	Adult	Juvenile	Adult
ERα	0.95 ± 0.45	0.69 ± 0.28	0.29 ± 0.02***	0.54 ± 0.10*
ERβ	1.34 ± 0.64	0.70 ± 0.21	0.20 ± 0.12**	1.24 ± 0.47
PR	2.03 ± 0.29*	0.48 ± 0.08**	0.27 ± 0.13*	0.87 ± 0.44
Clu	0.75 ± 0.17	0.93 ± 0.13	0.54 ± 0.24	0.79 ± 0.13
IGF-1	0.67 ± 0.23	1.34 ± 0.28	0.29 ± 0.03***	0.90 ± 0.22
IGF-1R	0.81 ± 0.32	0.60 ± 0.11	0.35 ± 0.12*	0.71 ± 0.24

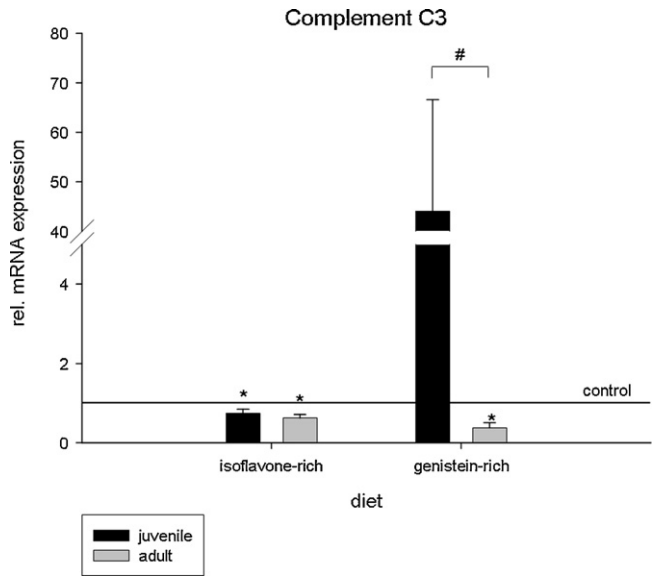

Fig. 3. Relative mRNA expression level of the *complement C3* gene after exposure to the respective diets (mean ± SD). Expression differs significantly from PE-free control group (black line): $^{*}p < 0.05$. mRNA level differs significantly between juvenile and adult animals in the same diet group: $^{\#}p < 0.05$. Both calculated with standard Student's *t*-test.

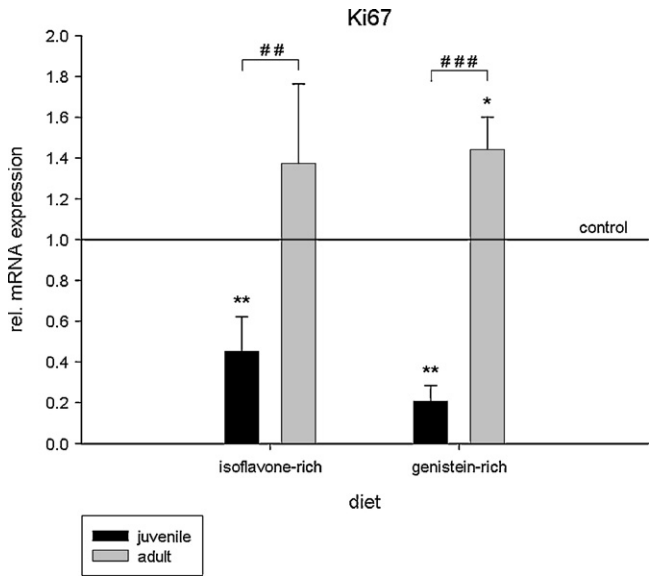

Fig. 4. Relative mRNA expression level of the proliferation marker *Ki67* gene after exposure to the respective diets (mean ± SD). Expression differs significantly from PE-free control group (black line): $^{**}p < 0.01$. mRNA level differs significantly between juvenile and adult animals in the same diet group: $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$. Both calculated with standard Student's *t*-test.

Only a weak but statistically not significant tendency of down-regulation could be seen in the GEN-rich diet group at day 21 (Table 3). An estrogenic response marker usually up-regulated following E_2 exposure is C3, a component of the complement cascade of the immune system [28]. A strong up-regulation (44.05 ± 22.53 fold) of C3 transcription could be detected in juvenile animals after feeding the GEN-rich diet. In adult animals, this up-regulation was completely reversed to a significant down-regulation (0.38 ± 0.13 fold) (Fig. 3). Also in the ISO-high diet group, a down-regulation (0.75 ± 0.09 fold) was already detected in the pre-puberty developmental stage and was maintained through adulthood (0.62 ± 0.09 fold) (Fig. 3).

An increased UWW can be attributed to numerous factors, such as proliferation and/or water imbibition. Therefore, we investigated the mRNA expression of genes associated with proliferation in the uterus. The two proliferation markers *PCNA* and *Ki67* responded in a similar expression pattern to the PE containing diets. Here again, the more sensitive time point was the juvenile stage. In juvenile uteri, both proliferation markers were down-regulated (PCNA: 0.47 ± 0.17 fold (ISO-high) and 0.53 ± 0.20 fold (GEN-rich); Ki67: 0.45 ± 0.17 fold (ISO-high) and 0.21 ± 0.08 fold (GEN-rich)) by both PE containing diets, with *Ki67* being the more sensitive one. Which means, at this developmental stage, exposure to both PE-diets resulted in anti-proliferative effects. However, in adult animals this effect was not detectable. In these animals, a weak up-regulation of *PCNA* (1.35 ± 0.06 fold (ISO-high) and 1.14 ± 0.18 fold (GEN-rich)) and *Ki67* expression (1.37 ± 0.39 fold (ISO-high) and 1.44 ± 0.16 fold (GEN-rich)) was observed, with *Ki67* being significantly induced by the GEN-rich and *PCNA* by the ISO-high diet (Figs. 4 and 5A). We additionally investigated the PCNA protein levels and confirmed mRNA expression data. Uterine protein samples were only available from adult animals. We detected similar PCNA protein levels in all dietary groups, although a slight induction (1.47 ± 0.99 fold) was observable in the ISO-high diet group (Fig. 5B).

The IGF-1/IGF-1R pathway is an important signalling pathway through which several biological effects of estrogens, amongst them proliferative responses, are mediated in the uterus. It thereby stimulates growth in many different cell types and concomitantly blocks apoptosis. In the case of the rat uterus, it has been known for almost 20 years that E_2 increases the concentration of *IGF*-1 as well as *type 1 IGF receptors* at the mRNA and protein levels [29,30]. Therefore, we investigated the effects of the diets on the expression profiles of *IGF-1* and *IGF-1R*. We observed that the GEN-supplemented diet led to a significant down-regulation of the *IGF-1* (0.29 ± 0.03 fold) and *IGF-1R* (0.35 ± 0.12 fold) mRNA levels in juvenile animals but these effects vanished in the adult OVX animals. ISO-high diet seems to have no effect on the regulation of the *IGF-1* gene expression at any of the investigated time points. In addition, the *type 1 IGF receptor* mRNA level was unaffected by the ISO-high diet at juvenile age, whereas it was weakly but significantly down-regulated in the adult (Table 3).

Table 4
Summary of results of proliferation related parameters in the rat uterus. Displayed are the changes of uterine wet weights (UWW) as well as the changes of proliferation marker mRNA levels. The arrows symbolize a decrease (↓), an increase (↑) or no change (↔) of the respective parameter compared to the PE-free control group. UWW ($^{a}p < 0.05$) and mRNA levels ($^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$) differ significantly from the PE-free control group.

Proliferation parameters	ISO-high		GEN-rich	
	Juvenile	Adult	Juvenile	Adult
UWW	↔	↔/↑^{a}	↑^{a}	↔/↑^{a}
PCNA	↓*	↔/↑	↓*	↔/↑
Ki67	↓**	↔/↑	↓**	↔/↑
IGF-1	↔	↔	↓***	↔
IGF-1R	↔	↔/↓	↓*	↔

4. Discussion

The possibility that the continuous ingestion of soy-rich food might have an impact on human health firstly attracted widespread attention almost 20 years ago. Since then, many publications have addressed these potential chemoprotective effects of ISOs on various outcome parameters. Several studies indicate that the East Asian diet, or constituents contained therein, has beneficial effects on the human organism but also contrary data exists. Therefore, we designed an animal study using three different diets to mimic commonly used nutritional habits: the PE-free diet (typical Western

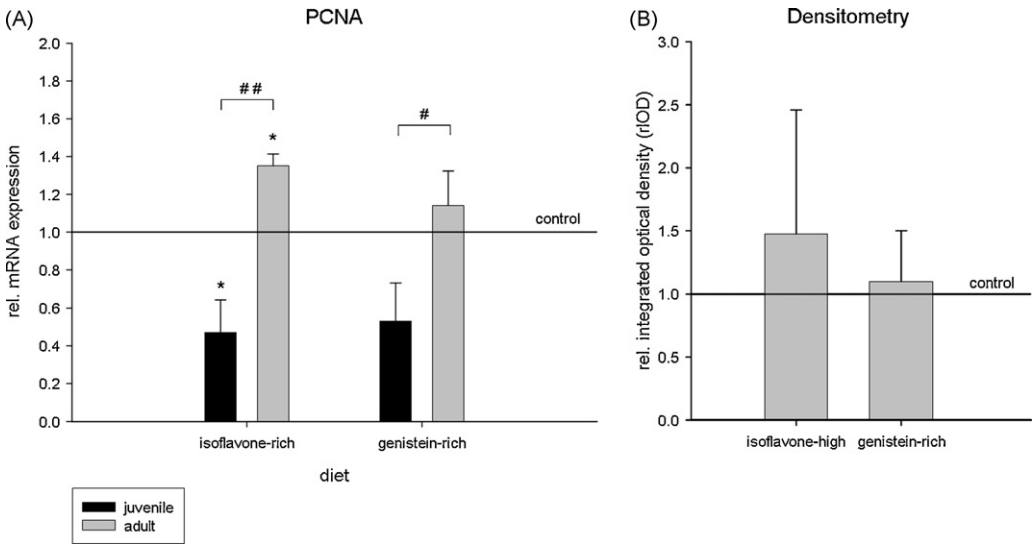

Fig. 5. (A) Relative mRNA expression level of the *PCNA* gene after exposure to the respective diets (mean ± SD). Expression differs significantly from PE-free control group (black line): $^{*}p < 0.05$. mRNA level differs significantly between juvenile and adult animals in the same diet group: $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$. Statistical significances are calculated with standard Student's *t*-test. (B) Densitometric analysis of PCNA protein levels after Western blot analysis of uterine tissue of adult ovariectomized animals after long-term exposure to the respective diets (mean ± SD).

diet), the ISO-high diet (traditional East Asian diet) and the GEN-rich diet (Western diet supplemented with GEN). In our study, we analyzed effects of lifelong exposure to phytoestrogens by starting the exposure *in utero* and maintained it continuously through the whole lifespan thereby investigating two different developmental stages, juvenile and adult. First, we chose immature rats (21 days of age) which were pre-pubertal and therefore not yet been exposed to high endogenous estrogen levels. These animals are a well-described sensitive model for uterotrophic responses [31,32]. In the second part of our experiment, mature adult rats at 97 days of age were used. These animals were ovariectomized to minimize the endogenous hormonal background. This procedure is necessary firstly to imitate the status of postmenopausal women and secondly to visualize potentially weak effects of the dietary ISOs, which would otherwise be covered by the endogenous hormonal status.

The uterus is one of the major target tissues of endogenous and exogenous estrogens [27]. Our results showed that a continuous exposure to GEN, which started prenatally, led to a duplication of the UWW in immature animals, whereas there was no difference in response to ISO-high diet compared to the PE-free control group (Fig. 2). In the respective adult OVX animals, the UWW increased by between 36 and 47% compared to the control group in response to GEN-rich or ISO-high dietary exposure respectively (Fig. 2). Although the results of our study showed substantially higher increases of the UWW, they are in line with the outcome of a study performed by Phrakonkham et al., who described an UWW increase of 12–18% in OVX animals fed with ISO supplemented diets for 90 days starting at day 15 post-partum [33]. These unequivocal differences in weight increase indicate that the intrauterine started exposure to dietary ISOs results in enhanced uterine sensitivities of the female offspring. Especially the chronic exposure to high levels of GEN seems to have adverse effects on juvenile uteri. Recent findings demonstrated that the UWW was significantly higher after perinatal ISO exposure compared to an ISO-free group [34]. Our results support the theory, that the effectiveness of dietary ISOs is highly dependent on the onset and duration of exposure. Additionally, an intrauterine started exposure seems to strongly enhance the estrogenic potential of these substances.

This enhanced sensitivity is also observed upon altered gene expression and protein profiles. We used qPCR to investigate the expression of relevant steroid receptors such as *ERα*, *ERβ* and the *PR*. Regulations in mRNA expression levels of *ERα* and *ERβ*, induced by ER ligands, have frequently been used as a molecular marker of estrogenic activity [35]. In our study, the mRNA levels of *ERα* and *ERβ* were not affected by the ISO-high diet whereas the GEN-rich diet led to a significant down-regulation in juvenile animals. Furthermore, *PR* mRNA expression revealed a very exciting regulation pattern. In the year 1993 was described, that estrogen exposure increases the mRNA and protein levels of PR in the rat uterus [36]. As we found in juvenile animals, the ISO-high diet (i.e., containing DAI and GEN) resulted in a significant increase of the *PR* mRNA level. However, the exposure to the GEN-rich diet led to a decrease of *PR* transcription. Whereas in adults, the GEN-supplemented diet had no effect on *PR* mRNA levels while co-exposure in ISO-high diet showed a significant down-regulation (Table 3). These opposing regulation patterns may have different causes. One possible explanation might be the elevated equol levels in animals of the ISO-high diet group but not of the GEN-rich group. Equol is a potent estrogenic agonist which was shown to increase *PR* transcription significantly [37]. It is a metabolite of DAI so that consequent high equol levels could be mainly measured in the blood plasma of animals exposed to the ISO-high diet. The uteri of animals fed with the GEN-rich diet (i.e., equol is absent) showed decreased PR mRNA levels. Therefore, the detected alleviation can be directly attributed to the GEN in the diet. This would indicate anti-estrogenic properties of GEN in contrast to the clear estrogenic properties of equol. Certainly, the animals of the GEN-rich group had a substantially higher daily GEN intake compared to the animals of the ISO-high diet group.

Also, two reliable response genes, *C3* and *Clu*, were chosen to evaluate estrogenic potency of dietary PEs, because the expression of both genes follows clear regulation patterns. *C3* expression is highly up-regulated following exposure to estrogens, because binding of the hormone receptor complex to the estrogen-responsive elements (ERE) in the promoter region subsequently initiates activation of transcription [27,38–41]. While an up-regulation of the *C3* gene represents an estrogenic effect, a down-regulation implies anti-estrogenicity. In our study, both, the GEN-rich and the ISO-high diet, tended to result in down-regulation of *C3* transcription in the adult rat uteri. In juvenile animals, the GEN-rich diet resulted in strong up-regulation of *C3* mRNA levels, in contrast to the ISO-high diet. *Clu* expression is known to be strongly down-regulated by estrogens although its promoter region does not contain EREs

[42,43]. Additionally, it is described that PEs are also able to induce this down-regulation [42]. In juvenile animals fed to GEN-rich diet *Clu* mRNA levels are weakly down-regulated. Simultaneously, the same animals show highly increased *C3* mRNA levels. Taken together these findings are suggestive of GEN being an estrogenic agonist in juvenile rats that mediates its transcriptional modulation predominantly via EREs.

Moreover, *IGF-1* and its receptor, which are important paracrine mediators of proliferative responses in the uterus [44], were investigated on mRNA level. Westley and May described *IGF*-1 and *IGF*-1R as an estrogen-induced growth factor/growth factor receptor system, whose expressions are up-regulated following estrogen treatment [44]. In our study, the GEN-rich diet exerted significant effects in juvenile animals by down-regulating expression of both genes. This indicates anti-proliferative properties of lifelong GEN exposure at least in pre-pubertal animals.

Since proliferation is a very sensitive estrogenic parameter in the uterus, we investigated the proliferation markers PCNA and Ki67. Both followed a similar regulation profile with Ki67 reacting more sensitive. This is in accordance with our previous data showing that *PCNA* and *Ki67* mRNA levels follow different temporal patterns [35]. The *PCNA* mRNA level was transiently elevated 24 h after estrogenic treatment and diminished after 3 days [35]. Here, the sensitive juvenile uteri exhibited an anti-proliferative response for both, the ISO-high as well as the GEN-rich diet group (Figs. 4 and 5A). This inhibited proliferation stands in contrast to the observed increased UWW, especially to those detectable in juvenile animals fed to the GEN-rich diet (Table 4). In conclusion these observations suggest that the higher uterine weights are caused by other reasons, for example, by water imbibition [45].

In summary, in this study we provide a further set of data useful for the understanding of the mechanism of GEN action. We present evidence that chronic exposure to PEs during fetal and postnatal development differently affects the UWW and gene expression in juvenile and adult rats. Interestingly, exposure to a diet supplemented with pure GEN might have both, potential adverse and beneficial effects. Starting GEN exposure already *in utero* induces a relative strong uterotrophic response in juvenile animals. Additionally, it exerts estrogenic effects on gene expression of the ERs as well as of *C3* in juveniles and slightly induces proliferation in adults. On the other hand, GEN acts anti-proliferative and anti-estrogenic (i.e., *PR* and *IGF-1*/*IGF-1R* mRNA levels) in juveniles and on certain parameters also in ovariectomized adults (i.e., *C3* mRNA level). The accrued picture of the complex GEN action supports the hypothesis of GEN being an interesting candidate molecule for future investigations and as a potentially natural occurring selective estrogen receptor modulator (SERM).

In conclusion, juvenile individuals respond sensitive and more diverse to the PE containing diets, whereas adults obviously do not. Additionally, our findings clearly show differences in the effects of East Asian like diet if compared to a pure GEN-supplemented Western diet. This observation may be relevant with respect to the issue of the growing practice of enriching food with isolated and purified ISOs.

Declaration of interest

The authors have declared no conflict of interest.

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