ORIGINAL

Effects of genistein in combination with conjugated estrogens on endometrial hyperplasia and metabolic dysfunction in ovariectomized mice

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Abstract. Tissue-selective estrogen complex (TSEC), which combines a selective estrogen receptor modulator (SERM) with one or more estrogens, is a novel approach to menopausal therapy. It has been demonstrated that the phytoestrogen genistein (GEN) exhibits mixed estrogen receptor agonist and antagonist activity, suggesting that GEN may have potential for use as a natural SERM. We evaluated, for the first time, the effects of GEN, conjugated estrogens (CE), and their pairing effects as a TSEC treatment on estrogen-induced endometrial hyperplasia and metabolic dysfunction in ovariectomized (OVX) mice fed a high-fat diet. CE replacement prevented fat accumulation in the adipose tissue and liver, improved glucose homeostasis, and induced endometrial hyperplasia in OVX mice. GEN at 100 mg/kg showed CE mimetic effects in preventing ovariectomy-induced metabolic dysfunctions without endometrial stimulation. Combination treatments with CE and GEN prevented metabolic dysfunctions more strongly than CE alone, but at both low and high doses, GEN did not reverse CE-induced endometrial hyperplasia. In addition, we found that in a TSEC regimen, a typical SERM raloxifene maintains the metabolic benefits of CE while simultaneously protecting the endometrium in OVX mice. These findings indicate that GEN acts as an estrogen agonist in metabolic regulation, but has no SERM function in the uteri of OVX mice.

Key words: Genistein, Conjugated estrogen, Tissue-selective estrogen complex, Endometrial hyperplasia, Metabolic dysfunction

WITH the dramatic increase in life expectancy, women spend a significant part of their lives in age-related estrogen deficiency. Loss of endogenous estrogen production after menopause contributes not only to impaired reproductive functions, but also increases the risk of metabolic syndrome and type 2 diabetes [1]. Estrogen replacement therapy (ERT) in postmenopausal women has been demonstrated to effectively offset these menopausal symptoms [2-4]. However, the use of ERT can cause hormone-dependent cancer associated with breast or endometrial cell proliferation [5]. To avoid an abnormal proliferative response in the breast or uterus tissue, the combination of progestin or selective estrogen receptor modulators (SERMs) with estrogens has been strongly suggested [6, 7]. SERMs are compounds that exert estrogen receptor (ER) agonistic or antagonistic activity depending on the target

tissue. An ideal SERM would have estrogen mimetic effects on menopausal symptoms with ER neutral or antagonistic activity in the breast or endometrium [8].

As a novel approach to menopausal therapy, tissue-selective estrogen complexes (TSEC), a combination regimen of a SERM with estrogens, has been reported to provide mixed estrogen agonist and antagonist activity in both rodents and humans [9, 10]. The first TSEC approved by the US FDA (3 October 2013), consisting of bazedoxifene and conjugated equine estrogens (CE), provides the desirable effects of estrogens for menopausal symptoms and the bone while simultaneously protecting the breast and endometrium from estrogen stimulation without the need for a progestin [11]. However, there are still concerns over the potential safety of SERMs including bazedoxifene in humans, as reports of phase 3 studies show increased incidences of hot flashes, leg cramps, and venous thromboembolic events after SERM treatments [12, 13]. Thus, efforts to find safer and natural alternatives for SERMs for use in postmenopausal treatment are needed.

Phytoestrogens, plant derived substances that are structurally and functionally similar to estrogens, have

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both ER agonistic or antagonistic activity, and have therefore been likened to natural SERMs [14]. The soy isoflavone genistein (GEN) exhibits estrogen agonist activity in the bones mediated through both ER α and ER β [15], but estrogen antagonist activity in the uteri of intact female mice [16]. In a combination treatment of GEN with estrogen, like a TSEC, GEN has been shown to inhibit 17 β -estradiol (E2)-stimulated responses in cancer cells of the breast [17], ovary [18], and prostate [19]. These results suggest that GEN mimics the tissue-specific actions of SERMs on ER activation. However, there is currently limited information regarding the efficacy of GEN paired with estrogens in postmenopausal treatment *in vivo* models. To determine the effects of GEN as a natural SERM in a TSEC regimen for oral therapy, we investigated the effect of GEN paired with CE on postmenopausal metabolic disorders in ovariectomized (OVX) mice fed a high-fat diet (HFD). The question of whether GEN maintains the metabolic effects of estrogen while simultaneously preventing endometrial stimulation by estrogen replacement in a postmenopausal mouse model is an important focus of this study.

Materials and Methods

Animals and surgery

Female C57BL/6J mice (Orient Bio Inc., Seongnam-Si, Korea), 7 weeks of age, were housed with a 12-h light-dark cycle. After an acclimation period of 1 week, mice were randomly divided into 8 treatment groups as follows: 1) sham vehicle, 2) OVX vehicle, 3) OVX + CE, 4) OVX + GEN25, 5) OVX + GEN100, 6) OVX + CE + GEN25, 7) OVX + CE + GEN100, 8) OVX + CE + raloxifene (RAL). The RAL, a typical SERM drug, was used as a positive control. Mice were subjected to bilateral OVX or sham operation under anesthesia with 1.2% avertin solution (i.p.). At this time point mice were 8-week-old, an age at which regular estrous cycles occur [20]. Treatment with CE, GEN, RAL or vehicle was initiated 1 week after surgery. All compounds were administered by gavage using an esophageal cannula once daily for 6 weeks with vehicle (saline, 2% Tween 80, 0.5% methylcellulose), CE 0.5 mg/kg, GEN 25 mg or 100 mg/kg, CE 0.5 mg/kg + GEN 25mg/kg, CE 0.5 mg/kg + GEN 100 mg/kg or CE 0.5 mg/kg + RAL 10 mg/kg in saline. Premarin (CE 0.625 mg tablet, USP), which is widely used for ERT in postmenopausal women, and Evista (RAL 60 mg tablet, USP) were used for CE and RAL treatments,

respectively. Dosage selected for CE corresponded to the minimal dose that reverses OVX-induced uterine atrophy [21], while RAL was administered at a dose sufficient to reverse CE-induced uterine hypertrophy [22]. GEN (> 99% purity) was purchased from LC Laboratories (Woburn, MA, USA). All mice were fed with phytoestrogen-free HFD (TD04059, 52% Kcal from anhydrous milk fat, Harlan Teklad, Madison, WI, USA) and water *ad libitum* during the experimental period. At the end of the study, mice were euthanized by an overdose of avertin, and blood collected by cardiac puncture. All animal work was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of Korea University (Seoul, Korea).

Histological staining

Sections of the parametrial adipose tissue and liver were fixed in 10% formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E). The adipocyte area was traced and quantified in 300 cells per mouse using ImageJ software (National Institutes of Health, NIH Version v1.32j). The relative adipocyte number was calculated by dividing parametrial fat pad weight by the mean adipocyte size in each mouse (n = 4 per group) as previously described [23].

Liver triglyceride measurement

Analysis of hepatic triglyceride (TG) content was performed by tissue saponification in ethanolic KOH and neutralization with MgCl₂ as previously described [24]. Glycerol content was determined by enzymatic colorimetric methods using a commercially available kit (Sigma-Aldrich, St. Louis, MO, USA).

Physiological studies of glucose homeostasis

Random-fed blood glucose was measured between 9:00 am and 10:00 am using OneTouch Ultra 2 glucose meter (LifeScan, Inc., Milpitas, CA, USA). Oral glucose tolerance test (OGTT) was performed as described previously [25] with some modification. After an 8-h fast, a glucose load (2 g/kg) was administered orally. For insulin tolerance test (ITT), after a 6-h fast, mice received an i.p. injection of 0.5 U/kg human insulin (Humulin, Eli Lilly, Indianapolis, IN, USA) in PBS. During OGTT and ITT, blood glucose levels were measured from the tail vein at 15, 30, 60, 90 and

120 min after administration of glucose or insulin. The area-under the curve (AUC) for glucose was calculated for each group of animals during OGTT and ITT.

Real-time Q-PCR

Liver, skeletal muscle, and uterine tissues were homogenized in 1 mL of TRIzol reagent and then total RNA was isolated. Total RNA was reverse transcribed to cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster, CA, USA). cDNA was used as a template for the relative quantitation of selected target genes with pre-designed TaqMan primer/probe sets (Integrated DNA Technologies, Coralville, IA, USA). Each 20 μ L reaction mixture contained 100 ng cDNA, 2 $\times$ SensiFAST Probe Lo-ROX mix (Bioline, Taunton, MA, USA), and TaqMan primer/probe. All reactions were carried out in triplicate with the 7500 Real-Time PCR Systems (Applied Biosystems) under the following conditions: 95 $^{\circ}$ C for 2 min followed by 40 cycles of 95 $^{\circ}$ C for 10 s and 60 $^{\circ}$ C for 30 s. Results were expressed as a relative value after normalization to β -actin.

Statistical analyses

Data were analyzed by one-way ANOVA using SAS software for Windows release 9.2 (SAS Institute Inc., Cary, NC, USA) on the W32_VSHOME platform. One-way ANOVA with repeated measures was performed to assess mean differences in body weight between groups overtime. To test for differences in uterine weights among the treatment groups, analysis of covariance (ANCOVA) with final body mass as covariates was used. Homogeneity of regression assumptions of the ANCOVA model were tested and met in each analysis. The Least Squares Means option using a Tukey–Kramer adjustment was used for the multiple comparisons among the treatment groups. Data were presented as the mean $\pm$ SEM. *P* values < 0.05 were considered statistically significant.

Results

GEN does not reverse estrogen-induced endometrial hyperplasia in OVX mice

To elucidate the estrogen antagonist activity of GEN in the uterus, we measured uterine weights at the end of experiment (Fig. 1A and B). As expected, the OVX surgery induced uterine atrophy in mice, and CE replacement markedly increased the uterine weight

in an estrogen deficient OVX model. Treatments with GEN alone, at both low- and high-doses did not induce uterine growth, but when combined with CE, GEN did not prevent uterine growth in OVX mice. As a positive control of SERM drug, RAL significantly reversed CE-induced uterine growth. To monitor compound responses in the uterus, we next measured mRNA expressions of key enzymes known to mediate estrogen-stimulated endometrial proliferation: leukemia inhibitory factor (*LIF*), G protein-coupled receptor 105 (*GPR105*), and insulin-like growth factor-1 (*IGF1*) [26–28]. Consistent with uterine weights, all gene expressions were significantly increased by CE and CE+GEN compared to vehicle treatment in OVX mice, and GEN alone did not influence their expression levels (Fig. 1C–E). The preventive effect of RAL in endometrial proliferation was mediated by suppressing the gene expressions of *LIF* and *GPR105* but not *IGF1* in CE-treated mice. Thus, the data demonstrate that, in a TSEC regimen, GEN does not exhibit estrogen antagonistic activity in the uterus of OVX mice.

GEN paired with CE prevents adiposity in OVX mice

Estrogens and their cognate receptors play a critical role in adipose tissue development [29]. Compared to sham-operated mice, vehicle-treated OVX mice exhibited a progressive increase in body weight characterized by accumulation of visceral and subcutaneous adipose tissues and adipocyte hypertrophy (Fig. 2). Treatments with CE and high-dose GEN prevented body weight increase, adipose tissue accumulation, and adipocyte hypertrophy. The combination of CE and high-dose GEN showed the greatest impact of all treatments including TSEC with RAL. Although the low-dose treatment of GEN caused no decrease in body fat, it maintained the suppressive effect of CE on adiposity. These results indicate that whether alone or in combination with estrogen, GEN exhibits estrogen mimetic action in preventing adiposity.

GEN paired with CE improves hepatic lipid homeostasis in OVX mice

Consistent with the reported observation of increased non-alcoholic fatty liver disease in menopause [30], vehicle-treated OVX mice exhibited diffused microvesicular fat infiltration in hepatocytes. This observation is shown by histological analysis (Fig. 3A) and resulted from increased hepatic TG accumulation (Fig. 3B). Both microvesicular fat infiltration in hepatocytes and

Fig. 1 GEN does not reverse estrogen-induced endometrial hyperplasia in OVX mice.

Mice were subjected to sham or OVX surgeries and received the indicated sample treatments for 6 weeks. (A) Representative images of uteri from each treatment group. (B) Uterine weights. Means are adjusted for final body mass as covariate using the ANCOVA analysis. (C) *LIF* mRNA, (D) *GPR105* mRNA, and (E) *IGF1* mRNA expressions from uterus (RQ: relative quantity). Values represent means $\pm$ SEM ($n = 7$). Data were analyzed by one-way ANOVA with a Tukey-Kramer multiple comparison. *Significantly different from the OVX vehicle group (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

hepatic TG accumulation were significantly reduced by all treatments except low-dose GEN, and the most pronounced reductions were observed in the combination treatments with CE and GEN (Fig 3A and B).

Because estrogen is a powerful regulator of lipogenesis and fatty acid oxidation [31], we assessed the gene expressions of carcinoembryonic antigen-related cell adhesion molecule 1 (*CEACAM1*) and peroxisome proliferator-activated receptor gamma coactivator-1 α (*PGC1 α*), which are key enzymes in hepatic

lipogenesis [32] and mitochondrial β -oxidation [33], respectively. Consistent with hepatic TG content, vehicle-treated OVX mice showed reduced hepatic mRNA levels of *CEACAM1* and *PGC1 α* that were significantly increased by CE alone or with high-dose GEN (Fig. 3C and D). The *PGC1 α* mRNA levels showed a significant increase in both liver and muscle by only the combined treatment of CE and high-dose GEN compared to OVX vehicle (Fig. 3D and E). In addition, CE and GEN alone or in combination decreased hepatic mRNA

Fig. 2 GEN paired with CE prevents adiposity in OVX mice.

Mice were subjected to sham or OVX surgeries and received the indicated sample treatments for 6 weeks. (A) Body weight, (B) weight gains, and (C) weights of visceral and subcutaneous adipose depots ($n = 7$). (D) Representative H&E stained section of parametrial adipose tissue and distribution histogram of adipocyte size (scale bar = 100 μ m). (E) Average size of adipocytes and (F) relative adipocyte number ($n = 4$). Values represent means $\pm$ SEM. Data were analyzed by one-way ANOVA with a Tukey-Kramer multiple comparison. For body weight, the repeated measures were performed to assess mean differences between groups overtime. *Significantly different from other treatment groups except the OVX+GEN25 group (A) or significantly different from the OVX vehicle group (B-F) (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

Fig. 3 GEN paired with CE improves hepatic lipid homeostasis in OVX mice.

Mice were subjected to sham or OVX surgeries and received the indicated sample treatments for 6 weeks. (A) Representative H&E stained sections of liver (scale bar = 100 μm), (B) liver TG contents, (C) liver *CEACAM1* mRNA, *PGC1α* mRNA in (D) liver and (E) muscle, and *PPARγ* mRNA in (F) liver and (G) muscle (RQ: relative quantity). Values represent means $\pm$ SEM (n = 7). Data were analyzed by one-way ANOVA with a Tukey-Kramer multiple comparison. *Significantly different from the OVX vehicle group (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

expression of peroxisome proliferator-activated receptor γ (*PPAR γ*) that plays a key role in development of hepatic steatosis [34], although there was no effect in the skeletal muscle (Fig. 3F and G).

GEN paired with CE improves glucose homeostasis in OVX mice

Estrogen deficiency in vehicle-treated OVX mice caused an increase in fed blood glucose that was prevented by estrogen replacements including the combination with GEN (Fig. 4A). These hormone treatments in OVX mice enhanced the clearance of exogenous glucose during OGTT (Fig. 4B and C) but did not affect glucose clearance in response to exogenous insulin (Fig. 4D and E). Estrogen has been known to improve glucose homeostasis through its actions on the expression and translocation of glucose transporter 4 (*GLUT4*) in muscle [35]. Interestingly, our data showed that GEN enhanced the positive effects of CE on muscle gene expressions of *GLUT4* and *PPAR δ* , which are major regulators of glucose metabolism (Fig. 4F and G). RAL maintained the effects of CE in overall glucose homeostasis. It is noteworthy that GEN alone at 100 mg/kg dose prevented adiposity (Fig. 2) and hepatic lipid accumulation (Fig. 3) and improved glucose tolerance (Fig. 4) to an extent similar to CE. Moreover, these protective effects in metabolic abnormalities were more pronounced with the combination treatments of CE and GEN than with CE alone.

Discussion

Soy isoflavones, the most potent and common phytoestrogens, have been shown to be effective supplements for the treatment of menopausal symptoms in both rodents and humans [36-38]. Previous comparative studies with estrogens showed that GEN exhibited estrogenic action in preventing bone loss and cardiovascular dysfunction without undesirable uterine stimulation caused by estrogen replacement in OVX rodents [39, 40]. These reports support our results showing that GEN mimics estrogen in exhibiting metabolic regulation without uterine stimulation, as reflected by the uterine weight and estrogen-responsive gene expressions in uterus. However, the uterotrophic effect of GEN seems to be dose-dependent. Indeed, when GEN was administered at a dose higher than 100 mg/kg which was used in our study, the increases of uterine weight and steroidogenic gene expressions

were observed in GEN-treated OVX mice compared to OVX control mice [41]. Thus, GEN dosage may be an important determinant of the uterine safety profile for its use in postmenopausal women. Importantly, our data show that both low- and high-dose GEN does not reverse CE-induced uterine stimulation, indicating that GEN has no SERM function in the uterus when combined with CE. It has been demonstrated that GEN exhibits estrogen antagonist activity by inhibiting E2-stimulated responses in the cancer cell lines of breast, ovary, and prostate [17-19]. In contrast, Somjen *et al.* [42] showed that RAL but not GEN blocked E2-induced creatine kinase activity in the uterus of OVX rats. Along with these observations, our present findings indicate that GEN may have estrogen antagonist activity depending on target tissues, but not in the uterus. Since one of important goals of SERM development is to block undesirable uterine responses by estrogens, this suggests that GEN may not be suitable as a natural SERM for TSEC therapy.

Although GEN did not antagonize estrogen responses in the uterus, our results clearly showed that GEN maintains or enhances the effects of estrogen in the prevention of metabolic dysfunctions in OVX mice. The effects of estrogens in control of energy balance and glucose homeostasis are mediated primarily by ER α -dependent mechanisms in the periphery as well as in the central nervous system [43]. A comparative study of selective ER subtype activation revealed that E2 and ER α agonist but not ER β agonist decreased hepatic and muscular mRNA expression of *PPAR γ* which is a major regulator of energy homeostasis in HFD-fed OVX rats [44]. Similarly, our current data showed that GEN and CE alone or in combination decreased hepatic *PPAR γ* mRNA expression, implying that GEN and CE act *via* ER α in liver. In addition, although GEN has shown a high affinity for ER β [45], the effects of GEN in body fat reduction require ER α [46]. Indeed, dietary GEN showed a dose-dependent effect on body fat reduction in OVX wild type mice, but the effects were lost in OVX ER α knockout mice [46]. Thus, in our study, the potent metabolic regulations of GEN in combination with CE could be mediated preferentially by ER α -dependent mechanisms.

Interestingly, our data here showed that CE and GEN have a beneficial combination effect in increasing *PGC1 α* mRNA expression in liver and muscle with improved glucose tolerance. *PGC1 α* has been shown to be a powerful regulator of lipid and glucose

Fig. 4 GEN paired with CE improves glucose homeostasis in OVX mice.

Mice were subjected to sham or OVX surgeries and received the indicated sample treatments for 6 weeks. (A) Fed blood glucose at Week 2, 4, and 6, (B) glucose concentrations during OGTT at Week 4, (C) area under the curve (AUC) for OGTT, (D) glucose concentrations during ITT at Week 5, and (E) AUC for ITT. (F) GLUT4 mRNA and (G) PPAR δ mRNA from muscle (RQ: relative quantity). Values represent means $\pm$ SEM (n = 7). Data were analyzed by one-way ANOVA with a Tukey-Kramer multiple comparison. *Significantly different from the OVX vehicle group (* P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001).

homeostasis by promoting fatty acid oxidation in liver and muscle and glucose uptake in muscle [47, 48]. It also has been reported that *PGC1 α* increases *GLUT4* expression *via* promoter activation in cultured muscle cells and skeletal muscle [49, 50], and this may explain the highest increase in muscle *GLUT4* mRNA expression with *PGC1 α* mRNA in the combination treatment with CE and GEN in this study. In addition, the combination treatment with GEN but not CE alone, significantly increased muscle gene expression of *PPAR δ* , an important regulator of glucose homeostasis and insulin sensitivity [51]. Kramer *et al.* [52] showed that activation of *PPAR δ* results in a direct activation of glucose transport in primary human myotubes. Together, these results suggest that GEN enhances the peripheral effects of estrogen for the improvement of glucose homeostasis through modulating gene expressions of key regulator enzymes in glucose metabolism. However, unexpectedly we did not observe the effects of estrogen or TSEC on insulin-stimulated glucose clearance during ITT, and further studies employing euglycemic-hyperinsulinemic clamp are needed to investigate the effects of estrogen combined with GEN in whole-body and tissue-specific glucose fluxes.

SERMs have been intensively studied over the past decade and have proven to be a promising agent for the treatment of postmenopausal conditions including hormone responsive cancer and osteoporosis. We recently demonstrated that TSEC consisting of CE and a third-generation SERM bazedoxifene effectively prevents metabolic dysfunction without uterine hypertrophy in OVX female mice [53]. Similar to bazedoxifene, RAL alone or in combination with CE has been shown to prevent osteoporosis and inhibit estrogen-induced uterine stimulation in OVX rodents and postmenopausal women [54, 55]. However, there is a lack of data on the metabolic effects of TSEC with RAL in peripheral tissues for postmenopausal treatment. Here, we showed that RAL maintained the metabolic benefits of CE in preventing adiposity, hepatic steatosis, and glucose intolerance as well as offset CE-induced uterine stimulation in OVX mice. These observations may help extend the therapeutic application of TSEC with RAL to postmenopausal obesity and diabetes. Further questions about the long-term benefits of RAL therapy in preventing postmenopausal metabolic abnormalities remain to be answered.

Enhanced tissue-selectivity of RAL on ER binding

is determined by its unique side chain structure (the imposition of a carbonyl “hinge” between the basic amine-containing side chain and the olefin) [56]. This antiestrogenic side chain is not present in phytoestrogens and this structural deficit may therefore limit their function as SERM [14], as shown by GEN in the present study. In recent years, there has been growing evidence that naturally occurring prenyl (a five-carbon side chain) substitution result in a tissue-specific antiestrogenic action from isoflavones, so-called phytoSERMs [57]. In most phytoSERMs studies, their antiestrogenic activities have been evaluated alone but not with estrogens. However, SERMs or phytoSERMs could provide an ideal benefit-risk profile for menopausal therapy when used in combination with estrogens rather than alone, because in general, their estrogenic activities in body are weaker than those of estrogens. Thus, an ideal phytoSERM would be a tissue-specific antiestrogenic compound suitable for combination with estrogens.

In conclusion, our study demonstrated that GEN at 100 mg/kg exhibited estrogen-mimetic effects in preventing metabolic dysfunctions without endometrial stimulation in OVX mice. More importantly, the combination treatments of CE and GEN prevented estrogen deficiency-induced adiposity, hepatic steatosis, and glucose intolerance with a stronger impact than CE alone. For TSEC therapy, RAL maintained the metabolic benefits of CE without causing endometrial hyperplasia, while GEN did not protect the uterus from CE-induced stimulation. These observations provide a better understanding of GEN use with or without estrogens as a potential postmenopausal therapy, and suggest that further phytoSERM studies are needed to identify their SERM functions with estrogens for the development of novel TSEC combinations.

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Disclosure

None of the authors have any potential conflicts of interest associated with this research.

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