Quercetin Induces Dose-Dependent Differential Morphological and Proliferative Changes in Rat Uteri in the Presence and in the Absence of Estrogen

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ABSTRACT Quercetin could have profound effects on uterine morphology and proliferation, which are known to be influenced by estrogen. This study investigated the effect of quercetin on these uterine parameters in the presence and in the absence of estrogen. Ovariectomized adult female rats received peanut oil, quercetin (10, 50, and 100 mg/kg/day), estrogen, or estrogen + quercetin (10, 50, or 100 mg/kg/day) treatment for 7 consecutive days. At the end of the treatment, uteri were harvested for histological and molecular biological analyses. Distribution of proliferative cell nuclear antigen (PCNA) protein in the uterus was observed by immunohistochemistry. Levels of expression of PCNA protein and mRNA in uterine tissue homogenates were determined by Western blotting and real-time polymerase chain reaction, respectively. Our findings indicated that administration of 10 mg/kg/day of quercetin either alone or with estrogen resulted in decreased uterine expression of PCNA protein and mRNA with the percentage of PCNA-positive cells in uterine luminal and glandular epithelia markedly reduced compared with estrogen-only treatment. Changes in uterine morphology were the opposite of changes observed following estrogen treatment. Treatment with 100 mg/kg/day of quercetin either alone or with estrogen resulted in elevated PCNA protein and mRNA expression. In addition, the percentages of PCNA-positive cells in the epithelia, which line the lumen and glands, were increased with morphological features mimicking changes that occur following estrogen treatment. Following 50 mg/kg/day quercetin treatment, the changes observed were in between those changes that occur following 10 and 100 mg/kg/day quercetin treatment. In conclusion, changes in uterine morphology and proliferation following 10 mg/kg/day quercetin treatment could be attributed to quercetin's antiestrogenic properties, while changes that occur following 100 mg/kg/day quercetin treatment could be attributed to quercetin's estrogenic properties.

KEY WORDS: • *quercetin* • *uterine morphology and proliferation*

INTRODUCTION

QUERCETIN [2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one], the most widely distributed plant flavonoid is one of the major constituents of the human diet.¹ Quercetin is abundantly present in the outer skin of onions, apples, and berries.² This compound possesses a broad range of biological activities, including anti-inflammatory, anti-pathogenic, antioxidant, and immunomodulatory.³ Due to its ability to bind to the estrogen receptor (ER),⁴ quercetin has been categorized as a phytoestrogen.⁵ Apart from ER, quercetin was also reported to bind to the type II estrogen-binding site.⁶ This compound was also found to inhibit the aromatase⁷ and sulfotransferase⁸ enzymes and downregulates the estrogen-regulated genes, pS2 and cathepsin D.⁹

The uterus, under the influence of estrogen, undergoes extensive growth and proliferation and ultimately achieves adequate thickness in preparation for embryo implantation.¹⁰ Several plant estrogenic compounds such as genistein have been reported to induce morphological and proliferation changes in the uterus.¹¹ Despite its reported estrogen-like activities, the effect of quercetin on uterine morphology and proliferation is currently unknown. In this study, we investigated the effect of quercetin on these uterine parameters in the absence of estrogen and in the presence of estrogen, which mimics the sex steroid profiles in the first half of the reproductive cycle where estrogen predominates.

MATERIALS AND METHODS

Animal preparation

Three-month-old adult female Sprague-Dawley rats, weighing 225 ± 10 g, were housed in a clean and well-ventilated environment with standardized conditions (lights on for 12 h from 06:00 to 18:00 h; room temperature $24^\circ\text{C} \pm 2^\circ\text{C}$; with

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five to six animals per cage). Animals had free access to a soy-free diet (Harlan Laboratories, Rossdorf, Germany) and tap water *ad libitum*. All experimental procedures were approved by the Institutional Animal Care and Use Committee (ACUC), University of Malaya. Quercetin, estrogen, and peanut oil were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Bilateral ovariectomy was performed under isoflurane anesthesia. Animals were divided into eight groups, which received the following treatments for 7 consecutive days:

- Group 1: peanut oil (vehicle)
- Group 2: estrogen at 0.2 $\mu\text{g/mL/day}$
- Group 3: quercetin at 10 mg/kg/day
- Group 4: quercetin at 50 mg/kg/day
- Group 5: quercetin at 100 mg/kg/day
- Group 6: quercetin at 10 mg/kg/day with 0.2 $\mu\text{g/mL/day}$ estrogen
- Group 7: quercetin at 50 mg/kg/day with 0.2 $\mu\text{g/mL/day}$ estrogen
- Group 8: quercetin at 100 mg/kg/day with 0.2 $\mu\text{g/mL/day}$ estrogen

Treatments were started at least 21 days after ovariectomy.¹² Drugs were subcutaneously injected behind the neck scruff. Quercetin was dissolved in 0.1 mL dimethyl sulfoxide, which was serially diluted to achieve the desired final concentration before mixing with peanut oil. A day after the last injection, rats were humanely sacrificed and uteri were removed for histology, protein, and mRNA expression analyses.

Quantification of proliferative cell nuclear antigen mRNA by real-time polymerase chain reaction

Whole uterine tissues were kept in the RNAlater solution (Ambion; Life Technologies, Carlsbad, CA, USA) before RNA extraction. Total RNA was freshly isolated from rat uteri by using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA). The purity and concentration of RNA were assessed by 260/280 UV absorption ratios (GeneQuant 1300; GE Healthcare UK Limited, Buckinghamshire, United Kingdom). Two-step real-time polymerase chain reaction (qPCR) was used to evaluate gene expression using a TaqMan[®] RNA-to-CT 1-Step Kit (Ambion; Life Technologies), which is highly sensitive.¹³ Reverse transcription into cDNA was performed by using a high-capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA). Controls include amplification performed on samples identically prepared with no reverse transcriptase and amplifications performed with no added substrate.

The assay used (TaqMan-Rn01514538_g1) amplifies the 94 bp segment of proliferative cell nuclear antigen (Pcna). Target assay was validated *in silico* by using whole rat genome and *in vitro* by using whole rat cDNA to ensure that target sequences were detected (Applied Biosystems). Gapdh was used as the reference gene as it is the most stably expressed in the uterus throughout the estrus cycle.¹⁴ The PCR program included 2 min at 50°C for uracil-N-glycosylase (UNG) ac-

tivity, 20 sec, 95°C, activation of AmpliTaq gold DNA polymerase, 1 min denaturation at 95°C, 20 sec, and annealing/extension at 60°C for 1 min. Denaturing and annealing were performed for 40 cycles. All measurements were normalized using GenEx software (Multid Analyses AB, Göteborg, Sweden), followed by Data Assist v3 software from Applied Biosystems that were used to calculate the RNA fold changes. All experiments were carried out in triplicates. Data were analyzed according to the comparative Ct ($2^{-\Delta\Delta C_t}$) method.

Quantification of PCNA protein expression by Western blotting

Whole uterine tissues were snap-frozen in liquid nitrogen and were stored at -80°C before protein extraction. Following extraction with PRO-PREP solution (iNtRON Biotechnology, Seongnam, Korea), equal amounts of protein were mixed with a loading dye, separated, transferred onto a polyvinylidene fluoride membrane (Bio-Rad Laboratories, Hercules, CA USA), and blocked with 5% bovine serum albumin (BSA) for 90 min at room temperature. The membrane was exposed overnight to goat polyclonal PCNA primary antibody (sc-9857; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at a dilution of 1:200 in phosphate-buffered saline (PBS) containing 1% BSA and Tween-20. The blots were then rinsed thrice in PBS-T, 5 min each. The membranes were then incubated with anti-goat, anti-mouse, and anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.) at a dilution of 1:500 for 1 h. The membranes were rinsed and subjected to the Opti-4CN[™] Substrate Kit (Bio-Rad Laboratories) to visualize the protein bands. Vinculin (sc-5573) was used as a loading control. Photographs of the blots were captured by using a gel documentation system, and the density of each band was measured by using ImageJ software (version 1.46j; National Institutes of Health [NIH], Bethesda, MD, USA). The ratio of each band/vinculin was calculated and was considered as the expression level of the target proteins.

Uterine histology and detection of uterine PCNA distribution by immunohistochemistry

Uterine tissues were fixed in 4% paraformaldehyde overnight before processing. The tissues were then dehydrated through increasing concentrations of ethanol, cleared in chloroform, and blocked in paraffin wax. Tissues were cut into 5- μm sections, deparaffinized in xylene, and rehydrated in reducing concentrations of ethanol. Trisodium citrate (pH 6.0) was used for antigen retrieval. One percent H_2O_2 in PBS was used to neutralize endogenous peroxidase. For immunoperoxidase staining, sections were blocked with 5% BSA for 1 h to prevent nonspecific binding before incubation with goat polyclonal PCNA primary antibody (as above) at a dilution of 1:200 in 5% BSA overnight. After four times of rinsing with PBS, sections were incubated with biotinylated secondary antibody for 30 min at room temperature, and were then exposed to avidin and biotinylated HRP complex (Santa Cruz Biotechnology, Inc.) in PBS for another 30 min. The sites of antibody binding were

visualized by diaminobenzidine-HCl (Santa Cruz Biotechnology, Inc.), which gave dark brown stains. Sections were counterstained with hematoxylin for nuclear staining. In this experiment, nonimmune normal donkey serum was used as a negative control where no staining was observed.

For immunofluorescence, sections were blocked in 10% normal rabbit serum (sc-2338; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature before incubation with PCNA primary antibody (as above) at a dilution of 1:100 in PBS with 1.5% normal donkey serum (sc-2044; Santa Cruz). After three rinses with PBS, sections were incubated with donkey anti-goat IgG-fluorochrome-conjugated secondary antibody (sc-2024; Santa Cruz Biotechnology, Inc.) at a dilution of 1:250 in PBS with 1.5% normal blocking serum at room temperature for 45 min. The slides were rinsed thrice with PBS and were mounted with Ultracruz mounting medium (Santa Cruz Biotechnology, Inc.) and counterstained with DAPI to visualize the nuclei. All images were viewed under a Nikon Eclipse 80i camera attached to a light microscope (Olympus, Tokyo, Japan).

Measurement of fluorescence signal intensity

Immunofluorescence images were captured under standard conditions of illumination with the chosen voltage, and the photographs were taken at a fixed exposure time. Tiff images (1280×1024 pixels) were taken at objective lens magnification of 40×. By using the NIS-Element AR program (Nikon Instruments, Inc., Melville, NY, USA), exposure time and sensitivity were set before image capturing. At the outset of the session, the part of the slide with no tissue (blank field) was viewed under the microscope and auto white balance was performed. Areas of interest on the image were selected under hue-saturation-intensity mode and total counts (fluorescence spots) were obtained. The mean in-

tensity of the counts (which could be restricted) was determined. This represents the average amount of protein expressed in the tissue.

Statistical analysis

Statistical differences were evaluated by *t*-test and one-way analysis of variance. A probability level of $P < .05$ was considered as significant. *Post hoc* statistical power analysis was performed and all values obtained were $P > .8$, which indicate adequate sample size. Meanwhile, the Shapiro-Wilk test was performed to test for data normality with $P > .05$. For mRNA expression analyses, $n = 6$ samples were used, and for immunohistochemistry and Western blotting, $n = 4$ samples were used.

RESULTS

Effects of quercetin on uterine morphology and PCNA distribution

Effects on luminal size. Figure 1 shows the appearance of the uterus under different treatments. Following estrogen-only and 100 mg/kg/day quercetin treatment, the lining of the uterine luminal epithelia was highly folded. Moderately folded epithelial lining was observed following combined estrogen and 100 mg/kg/day quercetin treatment. Less folded epithelial lining was observed in estrogen or non-estrogen-treated rats receiving 10 mg/kg/day quercetin treatment. The size of uterine lumen in estrogen or non-estrogen-treated rats receiving 50 mg/kg/day of quercetin was higher than the size observed following 10 mg/kg/day quercetin treatment.

Table 1 shows quantitative analyses of the ratio of uterine luminal to outer uterine circumference in rats receiving

FIG. 1. Uterine morphological changes at different doses of quercetin. The lumen was the largest in estrogen-treated rats. A smaller lumen was observed in the rats receiving 10 mg/kg/day of quercetin. Following 100 mg/kg/day quercetin treatment, the lumen size was almost identical to the estrogen-treated rats. C, control; E, estrogen; E+10Q, estrogen + 10 mg/kg/day quercetin; E+50Q, estrogen + 50 mg/kg/day quercetin; E+100Q, estrogen + 100 mg/kg/day quercetin; Q10, 10 mg/kg/day quercetin; Q50, 50 mg/kg/day quercetin; Q100, 100 mg/kg/day quercetin. Scale bar = 50 μ m. L, lumen. Color images available online at www.liebertpub.com/jmf

TABLE 1. RATIO OF LUMINAL/OUTER UTERINE CIRCUMFERENCE

Group	Luminal/outer uterine circumference
Control	1.02 ± 0.07
E	1.79* ± 0.25
E + 10Q	0.43 [†] ± 0.06
E + 50Q	0.51 [†] ± 0.14
E + 100Q	1.39 [†] ± 0.09
Q10	0.55* [†] ± 0.06
Q50	0.64* [†] ± 0.15
Q100	1.32* [†] ± 0.17

The value represents mean ± standard deviation for six rats per group.

* $P < .01$ compared with the control group.

[†] $P < .01$ compared with the estrogen group.

C, control; E, estrogen; E + 10Q, estrogen + 10 mg/kg/day quercetin; E + 50Q, estrogen + 50 mg/kg/day quercetin; E + 100Q, estrogen + 100 mg/kg/day quercetin; Q10, 10 mg/kg/day quercetin; Q50, 50 mg/kg/day quercetin; Q100, 100 mg/kg/day quercetin.

different treatments. The highest ratio was observed in estrogen-treated rats, followed by estrogen and nonestrogen-treated rats receiving 100 mg/kg/day quercetin treatment. In estrogen or nonestrogen-treated rats, which received 10 mg/kg/day quercetin treatment, the ratio of luminal to outer uterine circumference was lower than the rats that received estrogen-only treatment ($P < .05$). Similar decrease in the luminal over outer uterine circumference ratio was observed in estrogen or nonestrogen-treated rats receiving 50 mg/kg/day quercetin treatment ($P < .05$ compared with estrogen-only treatment).

Effects on morphology and PCNA distribution in luminal epithelia. In Figure 2A, the epithelia lining the uterine lumen in rats receiving estrogen-only treatment appear thick and tall with a rectangular shape. In the control rats and in estrogen and nonestrogen-treated ovariectomized rats, receiving 10 mg/kg/day quercetin treatment, the epithelia lining the uterine lumen appear thin and small. Furthermore, thick and tall epithelia could be seen lining the uterine lumen and uterine glandular lumen in both estrogen and nonestrogen-treated rats receiving 100 mg/kg/day quercetin treatment. Several mitotic figures were seen in the epithelia of rats receiving estrogen-only and estrogen plus 100 mg/kg/day quercetin and 100 mg/kg/day quercetin treatment. In these rats, the presence of clumped cells is indicative of active proliferation. Smaller epithelial cells could be seen in the estrogen and nonestrogen-treated rats that received 50 mg/kg/day quercetin treatment (compared with treatment with 100 mg/kg/day of quercetin).

In Figure 2A, immunoperoxidase images showed the cells with their nuclei stained with PCNA in luminal and glandular epithelia, respectively. In rats receiving estrogen-only treatment, multiple nuclei were seen to have a positive staining. Similarly, multiple positively stained nuclei were also seen in estrogen and nonestrogen-treated rat uteri following 100 mg/kg/day quercetin treatment. Treatment with 10 mg/kg/day of quercetin to both estrogen and nonestrogen-treated rats resulted in a lesser number of nuclei that were positively stained. In estrogen and nonestrogen-treated rats

that received 50 mg/kg/day of quercetin, few positively stained nuclei could be seen in the luminal epithelia.

Table 2 shows quantitative analyses of the heights of luminal and glandular epithelia in rats receiving different treatments. Our findings indicated that the heights of luminal and glandular epithelia were the highest following estrogen-only treatment. Administration of 10 mg/kg/day of quercetin either alone or in combination with estrogen resulted in a significant decrease in the heights of the luminal and glandular epithelia compared with estrogen-only treatment. Administration of 100 mg/kg/day of quercetin to estrogen and nonestrogen-treated rats resulted in an increase in luminal and glandular epithelial heights compared with control; however, they were not significantly different compared with estrogen-only treatment. The heights of luminal and glandular epithelia following estrogen plus 10 mg/kg/day quercetin treatment were ~2-fold lower than the heights observed following estrogen-only and estrogen plus 100 mg/kg/day quercetin treatment. In estrogen and nonestrogen-treated rats receiving 50 mg/kg/day quercetin treatment, the luminal and glandular epithelial heights were higher than the rats receiving 10 mg/kg/day quercetin treatment, but were lesser than the rats receiving 100 mg/kg/day quercetin treatment.

Figure 2B shows the percentage of PCNA-positive cells in the epithelia lining the uterine lumen of rats receiving different treatments. Our findings indicated that the highest percentage of PCNA-positive cells was observed in estrogen-treated rats. Administration of 10 mg/kg/day of quercetin caused the percentage of PCNA-positive luminal epithelial cells to decrease by ~2-fold compared with estrogen-only treatment. In estrogen-treated rats, a slightly lesser decrease in the percentage of PCNA-positive cells was observed following 50 mg/kg/day quercetin treatment. In estrogen-treated rats that received 100 mg/kg/day quercetin treatment, the percentage of PCNA-positive cells was not significantly different compared with estrogen-only treatment.

Administering 10 mg/kg/day of quercetin to nonestrogen-treated rats resulted in a lower percentage of PCNA-positive cells in the epithelia lining the uterine lumen compared with estrogen-only treatment (~8-fold). In these rats, the percentage of PCNA-positive cells increases with increasing doses of quercetin. Following 50 mg/kg/day quercetin treatment, the percentage was nearly 4-fold higher compared with 10 mg/kg/day quercetin treatment, while following 100 mg/kg/day quercetin treatment, the percentage was ~6-fold higher compared with 10 mg/kg/day quercetin treatment.

Effects on morphology and PCNA distribution in glandular epithelia and stroma. In Figure 3A, a dense endometrial stroma could be seen in estrogen-only-treated rats. In rats receiving vehicle treatment (control), the stroma appears to be less dense. Treatment with estrogen or nonestrogen-treated rats with 10 mg/kg/day of quercetin resulted in a marked decrease in stromal density. However, the density of endometrial stroma in both estrogen and nonestrogen-treated rats receiving 100 mg/kg/day of quercetin was almost similar to the density observed following estrogen-only treatment. Moderate stromal density

FIG. 2. Morphological changes and distribution of proliferative cell nuclear antigen (PCNA) in uterine luminal epithelia (A) and percentage of PCNA-positive luminal epithelial cells in different experimental groups (B). In estrogen and 100 mg/kg/day quercetin-treated rats, tall and large epithelia were seen. Following 10 mg/kg/day quercetin treatment, smaller rounded epithelia were seen. Multiple PCNA-stained nuclei were seen in the epithelia of estrogen-treated rats and in rats receiving 100 mg/kg/day quercetin treatment. The percentage of PCNA-positive cells was the highest following estrogen treatment. Scale bar = 50 μ m. G, gland. Arrow pointing toward PCNA-positive cells. * $P < .05$ compared to C, † $P < .05$ compared to E. Color images available online at www.liebertpub.com/jmf

was observed in rats receiving 50 mg/kg/day quercetin treatment.

In estrogen-only-treated rats, the glands appear large and well formed with abundant epithelial cells. Following treatment with 10 mg/kg/day of quercetin to estrogen and non-estrogen-treated rats, the glands appear small and less well formed. Larger glands were observed following 100 mg/kg/day quercetin treatment to either estrogen or nonestrogen-treated rats. Changes in the height of glandular epithelia as shown in Table 2 have been described earlier.

In Figure 3A, immunoperoxidase images showed that multiple nuclei stained with PCNA were seen in the glandular epithelia of rats receiving estrogen-only treatment. Multiple positively stained nuclei were also seen in the uteri

of estrogen and nonestrogen-treated rats following 100 mg/kg/day quercetin treatment. Treatment with 10 mg/kg/day of quercetin to both estrogen and nonestrogen-treated rats resulted in lesser number of nuclei positively stained with PCNA compared with estrogen-only treatment.

In Figure 3B, the percentage of PCNA-positive nuclei in glandular epithelia was the highest in estrogen-only-treated rats. In estrogen-treated rats receiving 10 mg/kg/day quercetin treatment, the percentage of PCNA-positive cells was markedly reduced (nearly 2-fold lower compared with control). In these rats, the percentage of PCNA-positive cells was proportionally increased with increasing doses of quercetin. Following administration of 100 mg/kg/day of quercetin, the percentage was not significantly different

TABLE 2. SHOWING LUMINAL EPITHELIAL HEIGHT AND GLANDULAR EPITHELIAL HEIGHT IN DIFFERENT TREATMENT GROUPS

Group	Luminal epithelial height (μm)	Glandular epithelial height (μm)
Control	7.24 $\pm$ 1.56	7.04 $\pm$ 0.92
E	39.54* $\pm$ 2.43	16.23* $\pm$ 1.43
E+10Q	18.53 [†] $\pm$ 2.21	8.46 [†] $\pm$ 1.75
E+50Q	25.54 [†] $\pm$ 2.18	11.35 [†] $\pm$ 1.55
E+100Q	34.23 $\pm$ 3.21	13.68 $\pm$ 2.24
Q10	10.43* [†] $\pm$ 1.75	8.24 [†] $\pm$ 1.45
Q50	21.37* [†] $\pm$ 1.87	10.42* [†] $\pm$ 1.28
Q100	29.54* [†] $\pm$ 1.65	12.23* $\pm$ 1.34

The value represents mean $\pm$ standard deviation for six rats per group.

* $P < .01$ compared with the control group.

[†] $P < .01$ compared with the estrogen group.

from estrogen-only-treated rats. In nonestrogen-treated rats, treatment with 10 mg/kg/day of quercetin resulted in the percentage of glandular epithelial cells positively stained with PCNA to be significantly lower compared with estrogen-only treatment. The percentage of PCNA-positive cells was increased in proportion with an increase in the doses of quercetin, being the highest following 100 mg/kg/day quercetin treatment.

Levels of PCNA expression in uterine luminal and glandular epithelia. Figure 4A shows immunofluorescence images, which revealed the highest signals in the epithelia lining the uterine lumen and uterine glandular lumen in estrogen-only-treated rats. In estrogen and nonestrogen-treated rats receiving 10 mg/kg/day quercetin treatment, the levels of PCNA expression were low. Following 50 mg/kg/day quercetin treatment to estrogen and nonestrogen-treated rats, a slightly higher PCNA expression was observed in the uterine luminal epithelia and glands compared with 10 mg/kg/day quercetin treatment. In estrogen and nonestrogen-treated rats receiving 100 mg/kg/day quercetin treatment, high levels of PCNA expression were observed in the epithelia lining the uterine lumen and uterine glandular lumen.

Figure 4B shows quantitative analyses of the intensity of fluorescence signals in the uterine luminal epithelia of rats receiving different treatments. Our findings indicated that the levels of expression of PCNA were the highest in rats receiving estrogen-only treatment, which was nearly 3-fold higher than controls. In estrogen-treated rats receiving 10 mg/kg/day quercetin treatment, the levels of PCNA expression were significantly lower than estrogen-only treatment. In these rats, administration of 50 and 100 mg/kg/day of quercetin resulted in an increase in fluorescence signal intensity directly in proportion with quercetin doses. Meanwhile, in nonestrogen-treated rats, levels of expression of PCNA in the epithelia lining the uterine lumen were in proportion to the quercetin doses, that is, low following 10 mg/kg/day quercetin treatment and high following 100 mg/kg/day quercetin treatment.

Figure 4C shows the quantitative analyses of fluorescence signal intensity in uterine glandular epithelia of rats receiving

different treatments. Our findings indicated that the intensity of fluorescence signals was the highest in rats receiving estrogen-only treatment. In these rats, concomitant administration of 10 mg/kg/day of quercetin resulted in ~ 2 -fold decreases in the fluorescence intensity level. However, administration of 50 and 100 mg/kg/day of quercetin resulted in lesser reduction in the fluorescence signal intensity in the epithelia lining the uterine glandular lumen. In nonestrogen-treated rats, administration of 10 mg/kg/day of quercetin resulted in lower fluorescence signal intensity compared with estrogen-treated rats. In these rats, the intensity was increased in proportion with the increase in quercetin doses. The highest intensity levels were seen in nonestrogen-treated rats receiving 100 mg/kg/day quercetin treatment.

Effects of quercetin on uterine PCNA mRNA and protein expression

Effects on Pcn mRNA expression. In Figure 5A, the levels of *Pcn* mRNA expression in uterine homogenates were the highest in estrogen-only-treated rats. In these rats, significantly lower mRNA levels were seen following administration of 10 mg/kg/day of quercetin. However, no significant difference in the levels of mRNA was observed between estrogen plus 100 mg/kg/day quercetin and estrogen-only-treated rats.

In nonestrogen-treated rats, expression of *Pcn* mRNA was markedly lower in rats administered 10 mg/kg/day quercetin compared with estrogen-only treatment (~ 2 -fold). However, following administration of 50 and 100 mg/kg/day of quercetin, levels of *Pcn* mRNA were higher than 10 mg/kg/day quercetin treatment. In these rats, *Pcn* expression levels were highest following 100 mg/kg/day quercetin treatment.

Effects on PCNA protein expression. In Figure 5B, expression of PCNA protein in uterine tissue homogenates was the highest in estrogen-only-treated rats. In rats receiving estrogen treatment, administration of 10 mg/kg/day of quercetin resulted in reduced expression of PCNA protein (~ 3 -fold) compared with estrogen-only treatment. In these rats, no significant differences in PCNA protein expression levels were observed following treatment with 50 mg/kg/day of quercetin compared with 10 mg/kg/day quercetin treatment. However, administration of 100 mg/kg/day of quercetin resulted in significantly higher PCNA protein expression levels compared with 10 mg/kg/day quercetin treatment.

In nonestrogen-treated rats, administration of 10 mg/kg/day of quercetin resulted in low expression levels of PCNA protein compared with estrogen-only treatment. Administration of 50 and 100 mg/kg/day of quercetin resulted in higher PCNA expression levels compared with 10 mg/kg/day quercetin treatment. In these rats, the levels of PCNA protein were the highest following 100 mg/kg/day quercetin treatment.

DISCUSSION

The findings from this study indicate that different doses of quercetin were able to produce different effects on the

FIG. 3. (A) Morphological changes and PCNA distribution in uterine glandular epithelia and (B) the percentage of PCNA-positive glandular epithelial cells in different experimental groups. In rats treated with estrogen and 100 mg/kg/day of quercetin, the epithelia appear tall and rounded. Smaller size of epithelia could be seen following 10 mg/kg/day quercetin treatment. The percentage of PCNA-positive cells was the highest in rats treated with estrogen. Scale bar = 50 μ m. M, mitotic figures. Arrow pointing toward PCNA-positive cells. * $P < .05$ compared to C, † $P < .05$ compared to E. Color images available online at www.liebertpub.com/jmf

uterus with or without estrogen influence. The effects observed following administration of 10 mg/kg/day of quercetin were the opposite of the effects produced following 100 mg/kg/day, and intermediate changes were observed following administration of 50 mg/kg/day of quercetin. At 10 mg/kg/day, quercetin caused a significant decrease in the endometrial thickness, the density of stroma, and the size of uterine lumen. Additionally, uterine glands were poorly formed with the luminal and glandular epithelia acquiring atrophic appearance. However, following 100 mg/kg/day quercetin treatment, the endometrial thicknesses were increased, the glands were well formed, the stroma was dense, and the epithelia were hypertrophied and hyperplastic. These effects resemble estrogen whereby endometrial thickness was

the greatest with glands that were well developed and the epithelia hypertrophied.¹⁰ Changes that occur in the uterus under 10 mg/kg/day quercetin influence could affect the normal endometrial morphology and therefore could interfere with the normal endometrial function and development.

We have shown that quercetin could also affect the proliferation of the uterine epithelia and stroma, as indicated by the changes in PCNA expression. Uterine proliferation was the highest under estrogen-only influence, as shown by the highest expression of PCNA protein and mRNA. Under the estrogen influence, active proliferation could be seen in the stroma and luminal and glandular epithelia as featured by the appearance of several mitotic figures and cell clumping, consistent with the previous findings.^{15–17} Positive PCNA

FIG. 4. (A) Immunofluorescence staining for PCNA in the uterus, (B) intensity of fluorescence signal in uterine luminal epithelia, and (C) uterine glandular epithelia in different treatment groups. High fluorescence signals were observed in rats receiving estrogen-only treatment and in rats receiving 100 mg/kg/day of quercetin. Low signals were seen in 10 mg/kg/day quercetin-treated rats. Green, site of antibody binding; blue, nuclei staining. Arrows point towards PCNA protein distribution. Scale bar = 50 μ m. * P < .05 compared to C, † P < .05 compared to E. Color images available online at www.liebertpub.com/jmf

cells were also seen along the lumen and glands. In addition, estrogen has also been reported to stimulate stromal fibroblast proliferation.¹⁸ Concomitant treatment of 10 mg/kg/day of quercetin with estrogen resulted in a marked decrease in the uterine PCNA protein and mRNA expression levels. In this group, the epithelial cells were small and atrophied. Similar changes were observed following administration of 10 mg/kg/day of quercetin to the rats not treated with estrogen.

The inhibitory effect of low-dose quercetin (10 mg/kg/day) on the uterine epithelial proliferation could be mediated either directly or indirectly through the stroma. Under the estrogen influence, the uterine stroma could produce several factors, including epidermal growth factor, which is

able to stimulate epithelial proliferation.¹⁹ Therefore, it was likely that quercetin inhibited the stromal estrogen effect, which would result in reduced proliferation and growth of the uterine luminal and glandular epithelia. Our hypothesis was supported by previous studies, which indicate that quercetin at 10 mg/kg/day could inhibit the estrogen effect on tissue growth and interfere with estrogen-induced protein expression.²⁰ Additionally, quercetin has also been reported to inhibit estrogen stimulation on the nuclear type II binding sites,²¹ which results in inhibition of uterine growth.²² Effects of quercetin on tissue growth were not only seen in the uterus but were also documented in the lungs.⁶ In the breast, low-dose quercetin has been found to inhibit ER-positive and ER-negative cancer through high affinity binding to the

FIG. 5. Uterine PCNA (A) mRNA and (B) protein expression in different experimental groups. The highest PCNA mRNA expression was observed in estrogen-only-treated rats. PCNA mRNA expression was reduced following administration of 10 mg/kg/day of quercetin. Similar changes were observed in PCNA protein expression. Molecular weight of PCNA = 36 kDa, vinculin = 117 kDa. * $P < .05$ compared to C, † $P < .05$ compared to E.

type II estrogen-binding site.²³ The effects of low-dose (10 mg/kg/day) quercetin could therefore be described as antiestrogenic. However, more work needs to be done to identify the pathways involved in mediating the inhibitory effect of 10 mg/kg/day of quercetin on estrogen-induced uterine growth and proliferation.

The findings from this study indicated that administration of 100 mg/kg/day quercetin to either estrogen-treated or nonestrogen-treated rats resulted in morphological and proliferative changes in the uterus, which resemble the effect of estrogen treatment alone. In view of this, it is likely that 100 mg/kg/day quercetin may bind to the ER, resulting in the activation of molecular pathways leading to the transcription of genes and expression of proteins that are

involved in proliferation. Our study has shown that the expression of PCNA mRNA was highly increased following 100 mg/kg/day quercetin treatment to either estrogen or nonestrogen-treated rats, which indicates that gene transcription was enhanced under quercetin influence. Additionally, expression of PCNA protein in the uterus and its distribution in the uterine luminal and glandular epithelia were also increased. Although quercetin was reported to have low affinity binding to ER,²⁴ we hypothesized that at this dose (100 mg/kg/day), quercetin could be able to stimulate the ER, resulting in increased cell growth and proliferation. However, further studies are needed to confirm the mechanisms underlying quercetin stimulation of uterine growth and proliferation. In the MCF7 human breast cancer cell

line, quercetin has been found to induce proliferation through binding to both ER- α and ER- β .²⁵

In conclusion, quercetin exerted both estrogenic and antiestrogenic effects on the uterus, depending on the doses. Several other flavonoid compounds such as kaempferide and apigenin were also reported to display both estrogenic and antiestrogenic effects, depending on the doses.²⁶ The observed quercetin effect could have an important implication on the reproductive processes. The antiestrogenic activity of quercetin could interfere with the uterine function and development. Therefore, quercetin could potentially be used as a contraceptive agent that prevents embryo implantation as well as to treat estrogen-dependent pathology such as uterine fibroid. On the other hand, at a high dose (100 mg/kg/day), quercetin could be used to replace estrogen, for example, in the therapy of postmenopausal estrogen deficiency. However, it should be applied with great precaution because at this dose, quercetin could potentially induce uterine hyperplastic and neoplastic changes.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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