Protective Effect of Quercetin on Endometrial Epithelial Barrier Disruption Induced by Bacterial Endotoxin

Sutthasinee Poonyachoti PhD*, Chatsri Deachapunya PhD**

** Department of Physiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand*
*** Department of Physiology, Faculty of Medicine, Srinakharinwirot University, Bangkok, Thailand*

Background: *Flavonol quercetin has beneficial effects for promoting epithelial barrier function in intestinal and airway epithelium; however, information is limited regarding its effects on the endometrium.*
Objective: *We investigated the potential effect of quercetin on epithelial barrier function and its protective effect on lipopolysaccharide (LPS)-induced disruption of barrier function in endometrial epithelial cells.*
Material and Method: *Immortalized porcine endometrial epithelial cells grown on porous membrane filters were treated with different concentrations of quercetin or LPS for 24 to 48 h. In experiments with LPS stimulation, the cells were treated with quercetin for 24 h prior to addition of LPS and further incubation for 24 h. The endometrial barrier function was assessed by measuring transepithelial electrical resistance (TER) and paracellular fluorescein isothiocyanate-dextran (FD-4) flux.*
Results: *Quercetin (1, 10 and 100 μM) concentration-dependently increased TER within the first hour reflecting increased membrane integrity, whereas high concentration (200 μM) significantly decreased TER at the first hour and persisted up to 48 h. However, the paracellular FD-4 transport was decreased by 1 and 100 μM quercetin. LPS exposure significantly disrupted endometrial barrier function as evidenced by decreased TER and increased FD-4 fluxes. This LPS-mediated barrier disruption was significantly reversed by pretreatment with quercetin at 1 and 10 μM.*
Conclusion: *Quercetin at concentrations less than 100 μM has a potential role in preserving endometrial epithelial barrier function. The reversibility effect of quercetin on endometrial barrier disruption induced by bacterial endotoxin could be considered of using quercetin to protect endometrial barrier against bacterial infection.*

Keywords: *Endometrial epithelium, Lipopolysaccharide, Tight junction, Transepithelial electrical resistance, Paracellular permeability*

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Endometrial epithelial cells lining the uterine cavity forms effective physical barrier that protects the body against invading micro-organisms and pathogen. The physical barrier is mainly composed of junctional protein complex including tight junction connecting between endometrial epithelial cells. These tight junction proteins are important structure correlated with barrier functions, which regulate the movement of ions and solute via a paracellular pathway. Moreover, the paracellular barrier function helps to maintain mucosal monolayer integrity that contributes to endometrial health. Disruption of tight junction of endometrial epithelial cells may result in the major complications in reproductive failure, such as infertility, infection and chronic abortion(1). A loss of epithelial tight junction barrier function following exposure to bacterial endotoxin, lipopolysaccharides (LPS) or *Clostridium perfringens* enterotoxin (CPE) has been evidenced in intestinal and endometrial epithelial cells(2,3). The leakiness of tight junction is associated with increased paracellular permeability as reflected by reduced transepithelial electrical resistance (TER) and increased movement of normally excluded molecules across the epithelium, resulting in inflammation and pathological conditions in reproductive system or any others.

At present, natural food components and compounds especially flavonoids have been identified to maintain or enhance membrane integrity and functions. Quercetin (3, 5, 7, 3′, 4′-pentahydroxyl-flavone) belongs to a subgroup of flavonols, and is the most potent dietary flavonoid found in high amounts in vegetable and fruits such as onions and apples(4,5). It possesses a variety of biological activities such as anti-bacterial, anti-inflammatory and potent anti-

Correspondence to:
Deachapunya C, Department of Physiology, Faculty of Medicine, Srinakharinwirot University, 114 Sukhumvit 23, Wattana, Bangkok 10110, Thailand.
Phone: +66-2-6495374, Fax: +66-2-2601533
E-mail: chatsri@gmail.com, chatsri@swu.ac.th

oxidative activities. It also exhibits beneficial effects in different human pathologies, including hypertension, cancer, and inflammatory conditions[6]. Quercetin has been reported to promote barrier function in rat intestines and human intestinal cell line[7,8]. It also possesses protective effects on cytokine-induced damage of intestinal tight junction barrier[7]. Moreover, it enhances tight junction (TJ) assembly and preserve TJ integrity and function against H_2O_2-mediated TJ disruption in human endothelial cell line[9]. These evidences indicate the protective role of quercetin on epithelial barrier function; although, the effect of quercetin on endometrial barrier function is quite limited. A recent study in endometrial epithelial cells demonstrates the ameliorative effect of soy isoflavones genistein and daidzein on the LPS-induced disruption of epithelial barrier protein and function[3].

The objectives of the present study were to investigate the potential effect of quercetin on endometrial barrier function and its protective role against bacterial endotoxin-induced alteration in barrier function using immortalized porcine glandular endometrial epithelial cell culture (PEG). The endometrial barrier function was assessed by measurements of TER and TJ permeability by fluorescein isothiocyanate-dextran (FD-4) transport across the paracellular pathway.

Material and Method

Materials

Quercetin, insulin, O111: B4 *Escherichia coli* lipopolysaccharide (LPS), non-essential amino acid and high purity grade salts were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate buffer saline (DPBS), fetal bovine serum (FBS), 0.05% trypsin-0.53 mM ethylene-diaminetetraacetic acid (EDTA), kanamycin and penicillin-streptomycin were purchased from GIBCO BRL (Grand Island, NY, USA).

Cell culture and treatment

Immortalized porcine endometrial epithelial (PEG) cells were primary glandular endometrial epithelial cells that were stably transfected with the catalytic subunit of human telomerase. These cells showed phenotypic and functional characteristics as the primary epithelial cells[10]. The PEG cell passage number 62 to 80 was cultured in DMEM supplemented with 3.7 g/L $NaHCO_3$, 10% heat-inactivated FBS, 5 μg/ml insulin, 1% non-essential amino acid, 100 U/ml penicillin, 100 μg/ml streptomycin and 100 μg/ml kanamycin (standard media), and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

PEG cells were cultured on porous polystyrene membrane inserts (1.12 cm^2, 0.4 μm pore size; Transwell Costar, Cambridge, MA, USA) for 7 days to obtain a complete monolayer. Quercetin (1, 10, 50, 100 or 200 μM) or vehicle control DMSO was applied to the solutions bathing both apical and basolateral membranes for 48 h. Each concentration of quercetin was dissolved in an equal volume of DMSO and then in a medium with a final concentration of DMSO at 0.01%. In the experiment with bacterial endotoxin stimulation, the cells were exposed to LPS (10, 100 or 1,000 ng/ml) for 24 h. To study the effect of quercetin under LPS stimulation, the cells were incubated with quercetin or vehicle DMSO for 24 h prior to addition of LPS (100 ng/ml) in the test medium for additional 24 h.

Cytotoxicity assay

The cytotoxic effect of quercetin on the PEG cells was assessed by a colorimetric assay using MTT [3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyltetrazolium bromide]. The cells were seeded in 96-well plates and incubated with different concentrations of quercetin for 24 to 48 h. The MTT assay is based on the mitochondrial metabolic reduction of soluble MTT to insoluble formazan crystals by mitochondria dehydrogenase of viable cells[11]. At the end of incubation period, the cells were incubated in DMEM containing MTT (125 μg/250 μl) at 37°C for 3 h in CO_2 incubator. The MTT medium was aspirated and the formazan crystals were dissolved in DMSO. The absorbance of formazan and non-specific background was respectively read at 570 nm and 620 nm by spectrophotometer (Tecan Sunrise™, Tecan trading AG, Switzerland). Relative cell numbers are proportional to the specific absorbance of formazan products, which are the subtraction of the 620 nm from 570 nm reading values.

Measurement of transepithelial electrical resistance (TER)

TER was performed to assess the permeability of tight junction or barrier function of epithelial cells. It was periodically measured using EVOM2™ electrode connected to a volt-ohmmeter (World Precision Instruments, Sarasota, FL, USA) before (0 min) and 0.5, 1, 2, 6, 12, 24 and 48 h after administration of quercetin or 0.5, 2, 6, 12 and 24 h after LPS inoculation. Percent changes of TER at each time point from the starting point (time 0) were calculated and analyzed. A decrease

in TER reflects an increase in tight junction permeability, whereas an increase in TER indicates an increase in monolayer tightening.

Measurement of paracellular permeability

The paracellular permeability of PEG cell monolayer was assessed by measuring the FITC-conjugated dextran fluxes across cell monolayer. For these experiments, FITC conjugated dextran of 4 kDa (FD-4; 1 mg/ml) prepared in HEPES buffered salt solution (HBSS): HEPES 25 mM, NaCl 120 mM, KCl 5.4 mM, $CaCl_2$ 1.8 mM, $NaHCO_3$ 25 mM, glucose 15 mM, pH 7.4 was added onto the apical side of the permeable membrane-grown PEG monolayers at 44 h of quercetin treatment or 20 h of LPS treatment. After incubation with FD-4 for 4 h at 37°C, media samples from the basolateral side were collected and fluorometrically determined at excitation 492 nm and emission 535 nm (Infinite® 200 PRO, Tecan, Mannedorf, Germany). The apparent permeability coefficients (P_{app}; cm/h) of different fluorescent agents used were calculated using the following equation:

$$P_{app} = \frac{dq}{dt} \times \frac{1}{A \times C^{\circ}}$$

Where dq is the amount of fluorescence in the basolateral side (mg/ml), dt is a function of time per hour, A is the surface area of the inserts (1.12 cm^2), and C^{o} is the initial concentration of fluorescent applied to the apical side (mg/ml).

Data analysis

All values are presented as mean±standard error of mean (SEM), and n is the number of monolayers used in each experiment. The statistical differences between control and treatment means were analyzed using an *ANOVA* and a Dunnette' post hoc test (Prism™ 5.00, GraphPad Software, San Diego, CA, USA). A *p*-value less than 0.05 was considered statistically significant.

Results

Effect of quercetin on PEG cell barrier function

The effect of quercetin on PEG cell was evaluated for barrier function by measurements of TER and the paracellular transport of FD-4 across cell monolayers. The PEG cells cultured in the media for 7 days showed an average TER of 1,376.3±70.56 $\Omega.cm^2$ (n = 80). In the experiments, the percent change of TER form the initial value before drug treatment (time 0) was calculated at each time point. Under control condition where the cells were replaced with a newly fresh medium in the presence of vehicle control DMSO, the TER remained unchanged for 0.5 h and slightly increased from initial at 1 h followed by a gradual decrease by 10 to 17% at 12 to 48 h (Fig. 1A). Treatment with 1 to 100 µM quercetin caused a concentration-dependent increase in the TER within 0.5 to 1 h and returned toward baseline at 2 h followed by a slow decrease in the similar profile as the control group. A significant increase in the TER was observed following

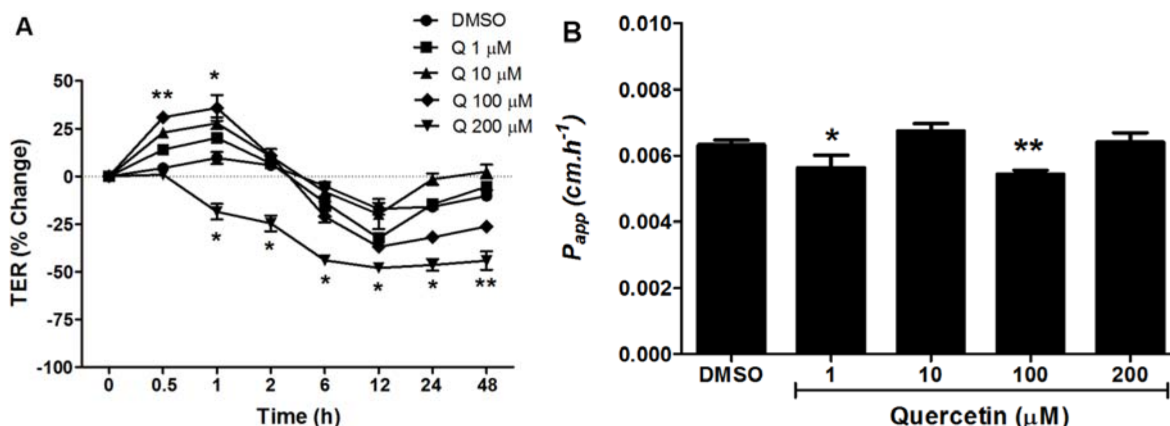

Fig. 1 Effects of quercetin (Q) on the tight junction permeability of PEG cells. (A) Time course of transepithelial electrical resistance (TER) as percent changes from initial (time 0) following quercetin treatment for 48 h. Data are mean ± SEM (n = 10-30). (B) The paracellular permeability of FITC-dextran (FD-4) calculated as apparent permeability coefficient (P_{app}) of FD-4 in response to quercetin treatment at indicated concentrations for 48 h. DMSO was used as a vehicle control. Data are mean ± SEM (n = 6). * $p<0.05$ and ** $p<0.01$ compared to DMSO at the same time point using ANOVA followed by Dunnett's post-hoc.

treatment with 100 μM quercetin during 0.5 to 1 h. However, the cells given quercetin at high concentration (200 μM) started to significantly decrease the TER by 25% at 1 h, reduced by 50% at 6 h and sustained up to 48 h.

To further assess the effect of quercetin on paracellular permeability, FD-4 which is commonly used as a marker of paracellular transport of macromolecules was additionally performed. The apparent permeability coefficient was used as an index for paracellular permeability of FD-4 from apical to basolateral sides of the PEG cell monolayer. As shown in Fig. 1B, the FD-4 permeability was significantly decreased by 1 or 100 μM quercetin, but not altered by 10 or 200 μM quercetin.

Effect of quercetin on cytotoxicity

Since the TER of PEG cell monolayer was decreased by quercetin at higher concentration, we then tested whether quercetin affected cell viability using MTT assay. Fig. 2 showed that addition of DMSO alone had no effects on viability of cells that were cultured in the standard media. Treatment with 1, 10 or 100 μM of quercetin for 24 and 48 h did not significantly affect cell viability. However, treatment with 200 μM quercetin for 48 h significantly decreased PEG cell viability as compared to the DMSO controls. Therefore, high concentration of quercetin (200 μM) was not used in the following LPS-challenged experiments while quercetin at 50 μM was additionally used.

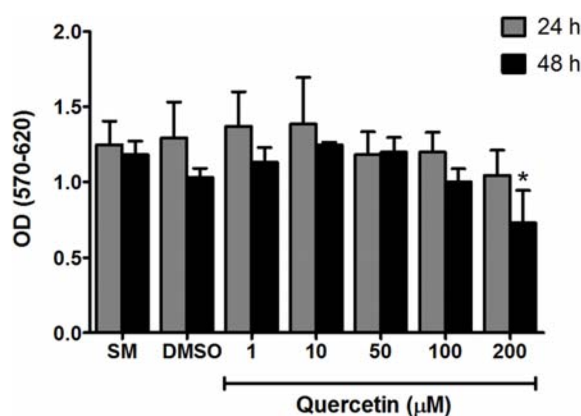

Fig. 2 Effects of quercetin on the viability of PEG cells. The cells were cultured in the standard media (SM) alone, or with DMSO or quercetin at indicated concentrations for 24 or 48 h. Cell viability was analyzed by MTT colorimetric assay. Values are expressed as mean±SEM (n = 4-5). Each experiment was done triplicate. * $p<0.05$ compared to DMSO at the same time point using ANOVA followed by Dunnett's post-hoc.

Effect of quercetin on PEG cell barrier function under LPS stimulation

Since LPS has been shown to impair the epithelial barrier function in the intestinal Caco-2 cell study[12], we further investigated the effects of LPS on barrier function and examined the quercetin effect on the LPS-induced epithelial barrier disruption in the endometrial epithelial cells. Fig. 3 showed that only 10 ng/ml LPS significantly increased TER whereas higher concentrations (100 and 1,000 ng/ml) had no effect on the TER within the first 30 min. However, following 2 h incubation, LPS produced a concentration-dependent decrease in the TER, reached maximal response at 6 h and sustained up to 24 h. Among different concentrations of LPS used, LPS at 100 ng/ml was chosen for the rest of the study as it reduced the TER by 50% from initial. After 24 h incubation with LPS, the TER was significantly decreased when compared to that of the control; however, pretreatment with quercetin at 1, 10 and 50 μM, but not 100 μM was found to suppress the LPS-induced decrease in TER (Fig. 4A). Likewise, the FD-4 fluxes which were significantly increased by LPS exposure relative to the control were significantly reversed close to the control values following pretreatment with 1, 10 or 100 μM quercetin (Fig. 4B).

Discussion

In the present study using immortalized porcine endometrial epithelial cells as a cell model, only

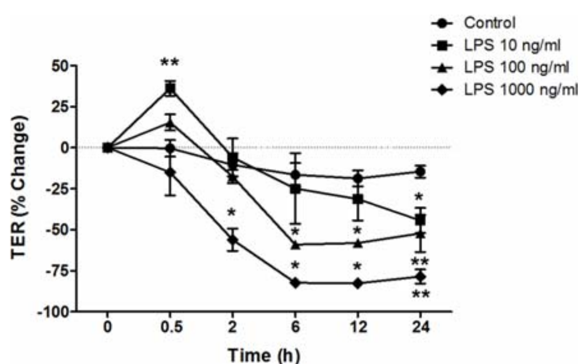

Fig. 3 Time course of TER as percent changes from initial (time 0) of PEG cells after stimulation with different concentrations of lipopolysaccharide (LPS) for 24 h. Data are mean ± SEM (n = 5). * $p<0.05$ or ** $p<0.01$ compared to control at the same time point using ANOVA followed by Dunnett's post-hoc.

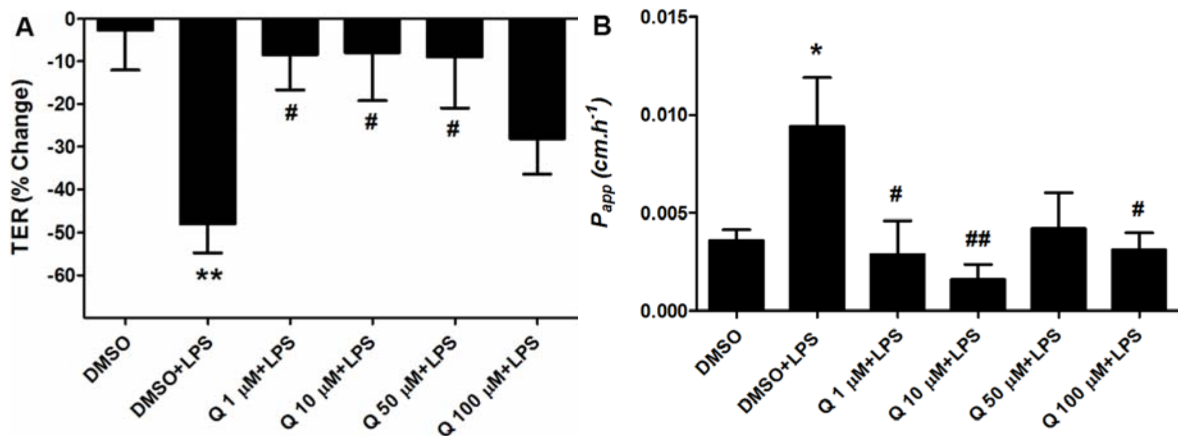

Fig. 4 Effects of quercetin (Q) on the tight junction permeability of LPS-stimulated PEG cells. (A) The percent change of TER and (B) the permeability coefficient (P_{app}) of FD-4 after stimulation with LPS 100 ng/ml for 24 h. The cells were pretreated with DMSO or quercetin at indicated concentrations for 24 h prior to LPS exposure. Data are mean ± SEM (n = 6). * p<0.05 and ** p<0.01 compared to DMSO and $^{\#}$ p<0.05 or $^{\#\#}$ p<0.01 compared to DMSO + LPS using ANOVA followed by Dunnett's post-hoc.

100 µM quercetin showed a significant increase in TER within the first hour, indicating the short-term effect of quercetin on promoting endometrial membrane integrity. However, high concentration of quercetin (200 µM) decreased the TER as early as 1 h and persisted up to 48 h. This finding contrasts the previous study in the human intestinal Caco-2 cell line reporting that the maximal increase in the TER was obtained with 200 µM quercetin[7]. In addition to cell type specific approach, the explanation for the different responses is likely due to high concentration of serum in those experiments (15% FCS) compared to our experiments (5% FBS). There has been evidence that quercetin that binds covalently to plasma protein could reduce the effect of 200 µM to 50 µM[7,13], and this serum effect may be responsible for the different TER responses by quercetin in a variety of experimental procedures. In the present study, a reduction in the TER with 200 µM quercetin treatment for 48 h may be partly due to the cytotoxicity of quercetin (Fig. 2).

Basically many pathogenic micro-organisms can regulate the tight junction barrier function by increasing or decreasing the tight junction barrier function[14]. The disruption of tight junction predominantly causes infertility and reproductive disorders. The bacterial endotoxin contamination via blood stream or ascending urogenital tract has been shown to primarily disrupt epithelial TJ barrier function[2,3]. Therefore, in the present study, the protective effect of quercetin on the decreased endometrial barrier function was further examined by challenging the PEG cells with bacterial endotoxin LPS following 24 h pretreatment with quercetin. In the present study, challenging the PEG cells with 10 to 1,000 ng/ml LPS caused a concentration-dependent decrease in TER (Fig. 3) which was consistent with reports of other studies in endometrial cells[15] or intestinal cell monolayers[16]. Pretreatment with 1 to 50 µM quercetin for 24 h prior to challenging with LPS inhibited the decreased TER induced by LPS (100 ng/ml) (Fig. 4A). This evidence suggests the tentative effect of quercetin on prevention of the disrupted TJ barrier function induced by bacterial endotoxin.

Besides the TER, the effects of quercetin on the unidirectional flux of FD-4 with a molecular weight of 4 kDa calculated as apparent permeability coefficient (P_{app}) were further tested for macromolecule transport across the paracellular pathway[17]. A decreased TER and increased FD-4 flux is suggested to associate with leaky epithelium[14]. In the present study, although 1 to 100 µM quercetin treatment for 48 h did not affect the TER, a slight decrease in the FD-4 flux reflecting tightening of TJ barrier function was observed with 1 and 100 µM quercetin. This finding indicates the effect of quercetin on preservation of endometrial barrier function. On the other hand, no change of the FD-4 flux in response to 200 µM quercetin was observed despite a significant decrease in the TER. As previously mentioned, a decrease in the TER by 48 h-treatments with 200 µM quercetin may be caused by the death of cells. The hyperpermeability has been normally indicated in the unhealthy and cell death[18]. However, the cell death induced by nutrient deprivation revealed the decreased TER with the decreased paracellular

permeability[19]. The cellular mechanism has been indicated that the cell injury during the nutrient deprivation induce the autophagic process leading to the suppression of pore-forming claudin-2 expression[18]. This results in the decrease of paracellular absorption of macromolecule, which may be associated with the decreased FD-4 flux. Similarly, the cell death correlated with the unchanged FD-4 flux in response to 200 μM quercetin may be explained by mechanism of autophagy. Since the autophagy pathway is to remove damaged and aged proteins and organelles to maintain cell homeostasis, the autophagy process induced by quercetin is of further interest.

Consistent with previous studies[12], cell exposure to LPS for 24 h not only decreased TER but also increased FD-4 flux, indicating its effect on disruption of endometrial barrier function. The present results with the FD-4 flux seem to be different from our previous study in the PEG cells[3] in which the FD-4 flux was not affected by LPS although a marked decrease in the TER. The increased FD-4 flux in response to LPS was indicated to be a dose- and time-dependent[20]. It is possible that longer LPS exposure time (48 h), and perhaps as yet unidentified factors, may account for the unresponsiveness of FD-4 flux by LPS in the previous study[3]. Thus, the decreased TER correlated with the increased FD-4 flux induced by 24-h LPS incubation in the present study serves as an appropriate model for inducing endometrial barrier disruption. Pretreatment with 1 to 100 μM quercetin for 24 h reversed the LPS-induced increase in the FD-4 flux, confirming the potential effect of quercetin on preventing tight junction barrier disruption induced by bacterial endotoxin.

Conclusion

The flavonol quercetin at concentration less than 100 μM has a potential role in preserving endometrial epithelial barrier function. Pretreatment with quercetin reversed the disruption of barrier function induced by bacterial endotoxin. These findings imply that quercetin may be used as barrier protective agent which helps protect the endometrial barrier function against bacterial infection.

What is already known on this topic?

Quercetin has been shown to preserve and enhance epithelial barrier integrity and function in intestinal and airway epithelia, but limited information in endometrial epithelial cells. It shows protective effect against barrier disruption induced by oxidative stress or cytokines.

What this study adds?

Quercetin at concentrations less than 100 μM preserves the epithelial integrity and barrier function in endometrium. It can protect against bacterial endotoxin-induced disruption of endometrial barrier function.

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Potential conflicts of interest

None.

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ฤทธิ์ของเควอซีทินต่อการทำหน้าที่ปกป้องความเสียหายของเยื่อบุผิวมดลูกที่ถูกเหนี่ยวนำโดยสารพิษจากแบคทีเรีย

สุทธาสินี ปุญญโชติ, ฉัตรศรี เดชะปัญญา

ภูมิหลัง: *สารฟลาโวนอลเควอซิทินออกฤทธิ์ส่งเสริมการทำหน้าที่ปกป้องร่างกายของเยื่อบุลำไส้และทางเดินหายใจ อย่างไรก็ตามการศึกษาผลของสารดังกล่าวในเยื่อบุผิวมดลูกยังมีอยู่อย่างจำกัด*

วัตถุประสงค์: *เพื่อศึกษาฤทธิ์สำคัญของเควอซีทินต่อการทำหน้าที่ปกป้องของเซลล์เยื่อบุผิวและผลของเควอซีทิน ในการป้องกันความเสียหายของเยื่อบุผิวมดลูกที่ถูกเหนี่ยวนำด้วยสารไลโปโพลีแซคคาไรด์ (lipolysaccharide, LPS)*

วัสดุและวิธีการ: *เซลล์เพาะเลี้ยงเยื่อบุมดลูกของสุกรชนิดไม่ตายที่เลี้ยงบนแผ่นค้ำจุนจะได้รับเควอซีทินหรือ LPS ที่ความเข้มข้นต่าง ๆ เป็นเวลา 24 ถึง 48 ชั่วโมง การทดลองภายใต้สภาวะที่ถูกกระตุ้นด้วย LPS เซลล์ได้รับเควอซีทิน เป็นเวลา 24 ชั่วโมงก่อนกระตุ้นด้วย LPS และบ่มต่อเป็นเวลา 24 ชั่วโมง การทำหน้าที่ปกป้องของเซลล์เยื่อบุมดลูกถูกประเมินโดยวัดค่าความต้านทานไฟฟ้าของเยื่อบุ (Transepithelial Electrical Resistance, TER) และการขนส่ง fluorescein isothiocyanate-dextran (FD-4) ผ่านช่องระหว่างเซลล์เยื่อบุ*

ผลการศึกษา: *เควอซีทินความเข้มข้น 1, 10 และ 100 μM เพิ่มค่า TER ตามความเข้มข้นที่เพิ่มขึ้นในช่วงเวลา 1 ชั่วโมงแรก ซึ่งสะท้อนถึงการเพิ่มความสมบูรณ์ของผนังเซลล์ แต่ที่ความเข้มข้น 200 μM กลับลดค่า TER ที่เวลา 1 ชั่วโมงและคงอยู่ถึง 48 ชั่วโมง อย่างไรก็ตามการขนส่ง FD-4 ผ่านช่องระหว่างเซลล์ลดลงเมื่อได้รับเควอซิทิน 1 และ 100 μM การสัมผัสกับ LPS ทำลายการทำหน้าที่ปกป้องของเซลล์เยื่อบุมดลูก ซึ่งเห็นได้จากการลดลงของ TER และเพิ่มการขนส่ง FD-4 ซึ่งผลของ LPS ถูกต่อต้านโดยการให้เควอซีทินความเข้มข้น 1 และ 10 μM ไปก่อน*

สรุป: *เควอซิทินที่ความเข้มข้นน้อยกว่า 100 μM มีศักยภาพดำรงรักษาการทำหน้าที่ปกป้องของเยื่อบุผิวมดลูก ผลของเควอซิทินในการต่อต้านการทำลายเยื่อบุมดลูกที่ถูกเหนี่ยวนำด้วยสารไลโปโพลีแซคคาไรด์อาจนำไปพิจารณาใช้เควอซิทินเพื่อปกป้องเซลล์เยื่อบุมดลูกจากการติดเชื้อแบคทีเรียได้*