Equol Production and Increased Leukocyte Mitochondrial DNA in Postmenopausal Women

Juwon Ahn1, Seungha Baek1, Kijeong Kim2, Hyowon Bang3, Jaehong Ko3, Jung-Ha Kim1

1Department of Family Medicine, Chung-Ang University Hospital, Chung-Ang University College of Medicine, Seoul, Korea
2Department of Microbiology, Chung-Ang University College of Medicine, Seoul, Korea
3Department of Physiology, Chung-Ang University College of Medicine, Seoul, Korea

Background: Equol, a metabolite of diadzein, is produced by some intestinal bacteria. Equol acts as an estrogen receptor agonist and has been reported to have several beneficial health effects. Leukocytes play an important role in the pathogenesis of autoimmune, metabolic, and cardiovascular diseases. Decreased leukocyte mitochondrial DNA (mtDNA) content, as an index of mitochondrial function, is associated with metabolic syndrome, bone mineral density, and aging. The possible association between equol production and leukocyte mitochondrial function has not been studied to date. Therefore, we investigated whether equol production is associated with leukocyte mtDNA copy number in postmenopausal women.

Methods: This observational cross-sectional study included 71 postmenopausal women. They completed a lifestyle questionnaire and medical history. In addition, a dietary assessment using a 24-hour recall method and food frequency questionnaire, anthropometric evaluation, and blood sampling were conducted. Serum equol concentration was measured in the fasting state. Leukocyte mtDNA copy number was measured by real-time polymerase chain reaction.

Results: Among older females, 33.8% were equol producers. The leukocyte mtDNA copy number was lower in non-equol producers versus equol producers. Furthermore, the leukocyte mtDNA copy number was positively associated with the serum equol concentration (r=0.42, \(P<0.01\)). Stepwise multiple regression analysis showed that equol production (\(\beta=47.864, P<0.01\)) was an independent factor associated with mtDNA copy number.

Conclusions: Equol production was associated with elevated mtDNA content in the peripheral blood of postmenopausal women. This finding suggests that the beneficial health effects of equol in postmenopausal women may be related to increased mitochondrial function.

Keywords: Equol, Leukocytes, DNA, Mitochondria, Postmenopause

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INTRODUCTION

Equol is a type of isoflavone metabolized from diadzein by bacterial flora in the intestines with soy consumption.1) However, not everyone can produce equol. Only about 20-59% of humans have been found to produce equol; and this percentage seems to be higher in Asians than in Westerners.2)
Recent studies found that the ability to produce equol is influenced by age, diet, and the use of antibiotics.\(^3\)

Equol has preferential binding affinity to estrogen receptors (ER)-β.\(^4\) Equol has been reported to relieve postmenopausal symptoms, have beneficial effects for prostate cancer, lower diastolic blood pressure in obese women, and prevent bone resorption.\(^5,6\) Furthermore, a recent study suggested that ER-β deficiency could lead to brain mitochondrial dysfunction and equol supplementation potentiates mitochondrial function in ovariectomized mice.\(^7\)

Leukocytes play an important role in the pathogenesis of autoimmune, metabolic, and cardiovascular diseases.\(^8\) Decreased leukocyte mitochondrial DNA (mtDNA) content, as an index of mitochondrial function, is associated with metabolic syndrome,\(^9\) bone mineral density, and aging.\(^10\) ER-β was also identified in human leukocytes, and equol production may influence leukocyte mitochondrial function such as the initiation of cellular signaling pathways and antioxidant effects.\(^11,12\)

However, the possible association between equol production and leukocyte mitochondrial function has not been studied to date. We investigated whether equol production is associated with leukocyte mtDNA copy number in postmenopausal women.

**METHODS**

This cross-sectional study included 71 postmenopausal women aged 64 to 83 years. They were recruited through flyers on a bulletin board at a hospital. All participants visited the primary healthcare clinic of Chung-ang University Hospital in Seoul for a medical examination and interview. None of the postmenopausal women had menstruated for at least 1 year, nor were they being treated with hormone therapy. Alcohol consumption was defined as consumption of 72 g or more of alcohol per week. Regular exercise was defined as physical exercise performed for at least 150 min/week. Past and current medical conditions and medication data were collected through individual surveys.

This study was approved by the institutional review board of Chung-ang University Hospital, and all participants provided written informed consent.

Anthropometric measurements were performed by a single, well-trained examiner. Body weight was measured to the nearest 0.1 kg using an electronic scale, with the participant in light clothing and no shoes. Height was measured to the nearest 0.1 cm using a stadiometer. Body mass index (BMI) was calculated as weight divided by height squared (kg/m\(^2\)). Waist-to-hip ratios (WHR), percent body fat, and lean body mass were estimated by the InBody720 (Biospace Co., Seoul, Korea). Blood pressure was measured in a seated position after a 10-minute rest period.

All blood samples for biochemical analysis were obtained after overnight fasting (>12 h). Serum levels of fasting glucose, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, and high-sensitivity C-reactive protein (hs-CRP) were measured using a Beckman Coulter AU5400 (Beckman Coulter Inc., Brea, CA, USA). Fasting insulin level and thyroid-stimulating hormone were measured by electrochemiluminescence immunoassay (ADVIA Centaur XP, Siemens Inc., Berlin and Munich, Germany). Insulin resistance was estimated by the homeostasis model assessment of insulin resistance (HOMA-IR) index [HOMA-IR=(insulin (μLU/mL) x fasting blood glucose (mg/dL)/18)/22.5]. Estimated glomerular filtration rate (eGFR) was calculated by the Modification of Diet in Renal Disease Study equation [eGFR (mL/min/1.73 m\(^2\))=175×(Serum creatinine)-1.154×(Age)-0.203×0.742].\(^13\) An electrochemiluminescence immunoassay (ARCHITECT, Abbott Diagnostics, North Chicago, IL, USA) was used to measure 25-hydroxyvitamin D (25-OH vitamin D) levels. Leukocyte counts were measured by the Sysmex XE-2100 (Sysmex Corp., Chuo-ku, Kobe, Japan).

Energy intake was analyzed through the CAN-Pro 4.0 program (The Korean nutrition society, Seoul, Korea) developed by the Korean Nutrition Society. Isoflavone intake was estimated using the soy food frequency questionnaire\(^14\) consisting of 14 food sources of isoflavone.

Plasma equol was extracted for time-resolved fluorescence immunoassay (TR-FIA)\(^15\) using an equol kit (Labmaster Ltd., Turku, Finland) according to the manufacturer’s instructions. Plasma equol glucuronide and sulfates were hydrolyzed by adding 200 mL of acetate buffer 0.1 M, pH 5.0, containing 0.2 U/mL of β-glucuronidase (Roche Diagnostics, Mannheim, Germany) and 2 U/mL of sulfatase (Sigma S9626, Sigma-Aldrich Inc., St. Louis, MO, USA) to tubes containing 200 mL of plasma. After mixing, the samples were incubated overnight at 37°C. On the following day, the free equol was extracted with 1.5 mL diethyl ether by careful mixing of the two phases for 3
minutes. The water phase was frozen in dry ice-ethanol mixture, and the ether phase was transferred into a disposable glass tube. After thawing, the water phase was re-extracted with ether, and the ether phases were combined and evaporated to dryness in a 45°C water bath. Assay buffer (200 mL) was added to the tubes; after careful mixing of 20 mL of the solution corresponding to 20 mL of the original plasma, the sample was taken for TR-FIA. The equol concentration of the sample was determined following the kit’s instructions and using a SpectraFluor Plus fluorescent plate reader (Tecan, GmbH, Austria) for fluorescence measurements.

Genomic DNA was isolated from 200 mL of the participants’ peripheral blood with DNeasy Blood & Tissue Kit (Qiagen Korea Ltd., Seoul, Korea). The DNA concentration and purity was measured by spectrophotometric absorbance at 260/280 nm using the Biotek Epoch microplate spectrophotometer (Winooski, VT, USA). The relative mtDNA copy number was measured by the quantitative real-time polymerase chain reaction (PCR)-based method as previously described, with some modifications. Briefly, for the mtDNA, the mitochondrially encoded subunit of complex I, ND5, was amplified, whereas, for the nuclear DNA (nDNA), the cystic fibrosis (CF) gene was used. ND5 primers were as follows: forward, 5′-AGGCGCCTATCACA CTCTGTTCG-3′ reverse, 5′-AACCTGTGAGGAAAGG TATCCTCG-3′. CF primers were as follows: forward, 5′-AGCAGAGTACCTGAAACAGGAA-3′ reverse, 5′-AG CTITACCATAGAGGAAACATAA-3′. The PCR reaction mixture (10 μL) for the mtDNA and nDNA amplification consisted of 1×PCR buffer, 0.2 mM dNTP, 3 mM MgCl2, 0.8 mg/mL BSA, 0.5× SYBR Green I dye (SIGMA S9430, Sigma-Aldrich Inc., St. Louis, MO, USA), 0.3 U ExTaq HS, 0.5 mM forward primer, 0.5 mM reverse primer, and 2 ng of genomic DNA for CF gene and 0.2 ng for ND5 gene. The thermal cycling conditions were 95°C for 3 min followed by 38 cycles at 95°C for 15s, 60°C for 20s, and 72°C for 40s. Each sample was run in duplicate in a 96-well optical plate. Real-time PCR was performed in the Light Cycler 96 (Roche Diagnostics, Mannheim, Germany).

A standard curve was made for both the mitochondrial and nuclear genes to calculate the number of copies of two genes in a given amount of DNA. The ND5 and CF genes were amplified from 0.04 to 25 ng of K562 DNA (Promega, Madison, WI, USA). The R² correlation for each standard curve was 0.99 or greater. Standard deviations for the quantification cycle value were accepted at 0.3. Otherwise, the test was repeated. The ratio of the mtDNA copy number to the single gene copy number was determined for each sample using standard curves. To test the inter-assay variation, the amplification of the ND5 and CF of a sample was tested using three different runs.

Data are presented as mean±standard deviation in a normal distribution, the median with interquartile range (25th-75th percentile) in a non-normal distribution or a number (%) in categorical variables. Triglyceride, hs-CRP, isoflavone intake, and mtDNA copy numbers were logarithmically transformed prior to statistical analyses to approximate a normal distribution.

The leukocyte mtDNA copy number was compared among premenopausal, postmenopausal non-equol producers (NP), and postmenopausal equol producers (EP) using the ANCOVA with adjusting for age. The baseline characteristics of the two groups (postmenopausal NP and EP) were compared using the t-test for continuous variables and Fisher’s exact test for categorical variables in cells with an expected count of less than five. For comparison among the three groups, ANOVA was used. Pearson’s correlation coefficients were calculated to evaluate the relationships between leukocyte mtDNA copy number and other variables, as well as between the leukocyte mtDNA copy number and the plasma equol concentration. Significance was defined at the 0.05 level of confidence. Stepwise multiple linear regression analysis was performed to identify factors that contributed to the leukocyte mtDNA copy number. Significance for entry into the model used the 0.15 level automatically determined in stepwise regression. All calculations were performed using the SAS 9.1 statistics package (SAS Institute Inc., Cary, NC, USA).

RESULTS

Table 1 shows the characteristics of the study participants according to postmenopausal NP and postmenopausal EP status.

The leukocyte mtDNA copy number was lower in NP than EP (P<0.01) (Fig. 1). Furthermore, the leukocyte mtDNA copy number was positively associated with serum equol concentration (r=0.42, P<0.01) (Fig. 2).

For univariate analyses using the Pearson’s correlation
Table 1. Characteristics of the study subjects (n=71)

<table>
<thead>
<tr>
<th></th>
<th>Equol producer (n=24)</th>
<th>Non-equol producer (n=47)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>71.6±4.4</td>
<td>69.9±3.7</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>25.6±2.8</td>
<td>24.7±3.2</td>
</tr>
<tr>
<td>Waist-hip ratio</td>
<td>0.9±0.1</td>
<td>0.9±0.1</td>
</tr>
<tr>
<td>Total body fat, %</td>
<td>35.6±6.2</td>
<td>34.5±7.0</td>
</tr>
<tr>
<td>Total body lean mass, kg</td>
<td>21.0±2.6</td>
<td>19.6±2.3</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>127.6±11.9</td>
<td>129.4±11.7</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>72.3±9.4</td>
<td>74.8±9.2</td>
</tr>
<tr>
<td>Fasting glucose, mg/dL</td>
<td>101.4±18.2</td>
<td>102.8±16.0</td>
</tr>
<tr>
<td>Fasting insulin, μIU/mL</td>
<td>6.9±3.0</td>
<td>6.7±2.9</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.7±0.0</td>
<td>1.7±0.8</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>199.2±41.2</td>
<td>202.5±46.3</td>
</tr>
<tr>
<td>Triglyceride, mg/dL</td>
<td>106.5 (64.0-131.0)</td>
<td>127 (81-127)</td>
</tr>
<tr>
<td>HDL-cholesterol, mg/dL</td>
<td>51.9±10.5</td>
<td>51.1±9.8</td>
</tr>
<tr>
<td>LDL-cholesterol, mg/dL</td>
<td>121.1±34.5</td>
<td>123.5±37.4</td>
</tr>
<tr>
<td>Estimated GFR, mL/min</td>
<td>97.5±19.7</td>
<td>93.8±17.4</td>
</tr>
<tr>
<td>Leukocyte, /μL</td>
<td>5,662.8±1,410.8</td>
<td>5,862.5±1,473.0</td>
</tr>
<tr>
<td>Hs-CRP, mg/mL</td>
<td>0.6 (0.3-1.3)</td>
<td>0.8 (0.4-2.6)</td>
</tr>
<tr>
<td>TSH, μIU/mL</td>
<td>1.9±1.1</td>
<td>2.2±1.1</td>
</tr>
<tr>
<td>25-OH vitamin D, ng/mL</td>
<td>12.5±5.2</td>
<td>12.2±6.1</td>
</tr>
<tr>
<td>Total energy intake, kcal/d</td>
<td>1,488.9±283.4</td>
<td>1,587.7±314.8</td>
</tr>
<tr>
<td>Protein intake, % kcal</td>
<td>15.9±2.0</td>
<td>16.0±2.7</td>
</tr>
<tr>
<td>Isoflavone intake, mg/dL</td>
<td>38.3 (13.9-59.5)</td>
<td>25.5 (13.4-41.0)</td>
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<tr>
<td>Current smoker</td>
<td>1 (4.2)</td>
<td>0</td>
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<tr>
<td>Alcohol consumption</td>
<td>4 (16.7)</td>
<td>6 (12.8)</td>
</tr>
<tr>
<td>Regular exercise</td>
<td>12 (50.0)</td>
<td>20 (42.6)</td>
</tr>
<tr>
<td>Medication</td>
<td></td>
<td></td>
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<tr>
<td>Hypertension</td>
<td>10 (41.7)</td>
<td>20 (42.6)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>3 (12.5)</td>
<td>7 (14.9)</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>4 (16.7)</td>
<td>8 (17.0)</td>
</tr>
</tbody>
</table>

Abbreviations: HOMA-IR, homeostasis model assessment of insulin resistance; HDL, high-density lipoprotein; LDL, low-density lipoprotein; GFR, glomerular filtration rate; Hs-CRP, high-sensitivity C-reactive protein; TSH, thyroid-stimulating hormone; 25-OH vitamin D, 25-hydroxyvitamin D.

Values are presented as mean±SD unless otherwise indicated.

Median (interquartile range, IQR).

*Defined as consumption of 72 g or more of alcohol per week.

analysis, the leukocyte mtDNA copy number was negatively correlated with age (r=-0.20, P=0.04), systolic blood pressure (r=0.26, P=0.01), and hs-CRP (r=-0.22, P=0.03) and positively correlated with eGFR (r=0.20, P=0.04) (Table 2).

Stepwise multiple regression analysis showed that equol production (β=47.864, P<0.01) was an independent factor associated with mtDNA copy number after adjusting for age; BMI; WHR; total body fat; total lean body mass; systolic blood pressure; eGFR, fasting glucose; hs-CRP, 25-OH vitamin D; intake of energy, protein, and isoflavone; alcohol consumption; regular exercise; and users of anti-hypertensive, anti-diabetic, and lipid lowering agents (Table 3).

In conclusion, postmenopausal EP have significantly higher leukocyte mtDNA copy number than postmenopausal NP. Moreover, the leukocyte mtDNA copy number seems to be positively associated with serum equol concentration in postmenopausal women. This finding suggests that the metabolite, equol, acts as a selective estrogen modulator, activating leukocyte mtDNA contents in postmenopausal women. Further research is necessary to determine the association between supplements and to identify other associated factors.

**DISCUSSION**

The results of this study indicate that EP have significantly higher leukocyte mtDNA copy number than NP among postmenopausal women. Moreover, the leukocyte mtDNA copy number is positively associated with serum equol concentrations. We also found that equol production was independently associated with the mtDNA copy number. The mechanism of equol in the regulation of mtDNA
content in human leukocytes is unclear. Recent studies suggested that equol acts as natural selective estrogen receptor modulators.\(^1^7\) Estrogen has multiple effects on mitochondrial function including regulating both nuclear and mitochondrial transcription, influencing the activation of signaling pathways, and having direct antioxidant effects.\(^1^1,1^2\) Equol might bind to ER-β and act similarly to estradiol,\(^4^0\) thereby increasing the leukocyte mtDNA content during menopause.

About 50-59% of Asian adults have been found to produce equol from diadzein,\(^2^3\) which is significantly higher than the reported 20-35% of adult Westerners. However,
only 33.8% of participants in this study were EP, which is lower than that reported in similar studies. There may be several reasons for this difference. First, we recruited postmenopausal versus adult women, making our subjects older. At least one study reported that the EP percentage decreased with increasing age.\(^{18}\) Second, in this study, we did not control the uptake of isoflavone. Postmenopausal NP consumed less isoflavone than postmenopausal EP, even though there was no difference in total energy and protein intake. Two studies reported that long-term soy ingestion could convert NP to EP.\(^3,14\) Thus, if NP consumed enough isoflavones, they might acquire the ability to produce equol.

According to a previous study, the average daily intake of isoflavone in postmenopausal women in Korea is 21.94 mg.\(^{19}\) So far, there has been no recommended daily intake of isoflavones. Although it is necessary to take 50 mg of isoflavone to relieve postmenopausal symptoms,\(^{14}\) the amount of isoflavone needed to produce equol from diadzein has not been determined. Considering the high population ratio of EP observed in Asia, especially Taiwan where soybean-containing foods are generally consumed,\(^{18}\) we should encourage the intake of more isoflavones.

The design and results of this study may raise some discussions. First, we did not perform biopsies of the target tissue, skeletal muscle, or fat, which is considered the gold standard for evaluation of mitochondrial function. However, it has been suggested that the mtDNA copy number of peripheral blood may reflect that of muscle and live tissue in rats.\(^{20}\) Second, we did not examine serum estradiol levels in participants. Since we enrolled women who had not menstruated for at least 1 year, serum estradiol level would not be that different among the participants. Third, with cross-sectional studies, it is difficult to identify the cause and mechanism between the leukocyte mtDNA copy number and equol production. Finally, because the study was not conducted in the community, there may be selection bias.

**요약**

**연구배경:** 이퀄(equol)은 다이아세인(diadzein)이 장내 미생물에 의해 대사되어 만들어지는 대사물로서, 모든 사람에게서 생성되지는 않는다. 이퀄은 에스트로겐 수용체에 작용하여 배경기 증상을 줄이고, 골다공증과 전립선 암, 심혈관 질환 등에 긍정적인 영향을 미치는 것으로 알려져 있다. 그리 고 백혈구는 면역, 대사, 심혈관 질환에서 중요한 역할을 담당한다. 미토콘드리아 기능을 측정하는 수치인 백혈구 미토콘드리아의 DNA 수가 감소하면, 대사질환, 골다사, 노화가 진행하는 것으로 알려져 있다. 그러나 현재까지 이퀄 생성과 백혈구 미토콘드리아의 기능의 관계에 대한 연구는 없었다. 따라서 이 논문에서는 폐경 여성에서의 이퀄 생성과 백혈구 미토콘드리아의 DNA 수에 관계에 관한 연구를 하고자 한다.

**방법:** 이 관찰 단면연구는 71명의 폐경 여성 대상으로 한다. 생활습관에 관한 설문지, 병력에 관한 설문지를 작성하였고, 24시간 기여화험법과 섭취빈도법을 이용하여 식이분석, 신체계측, 혈액검사를 시행하였다. 이퀄 생성은 공복 혈당의 이퀄 농도로 결정하였고, 백혈구 미토콘드리아의 DNA수는 실시간 중합효소연쇄반응으로 측정하였다.

결과: 폐경 여성에서 이퀄 생성자는 33.8%였다. 이퀄 생성 여성보다 이quil 생성 폐경 여성의 백혈구 미토콘드리아 DNA수는 향상되었고, 혈중 이퀄 농도와 양의 상관관계를 가지고 있었다 (r=0.42, P<0.01). 다중회귀분석에서 미토콘드리아 DNA수와 이퀄의 생성은 독립적으로 관련이 있었다.

**결론:** 이퀄 생성은 폐경 여성의 말초혈액에서 미토콘드리아 DNA 양의 증가와 관련이 있다. 이런 결과는 이퀄이 건강에 미치는 긍정적인 효과가 미토콘드리아의 기능과 관련되어 있을 수도 있음을 시사한다.

**중심 단어:** 이퀄, 백혈구, DNA, 미토콘드리아, 폐경

**REFERENCES**


