

# Shock: Injury, Inflammation, and Sepsis: Laboratory and Clinical Approaches

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**Equol, a soybean metabolite with estrogen-like functions, decreases  
lipopolysaccharide-induced human neutrophil extracellular traps *in vitro***

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Running head: Equol decreases LPS-induced NETs

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**ABSTRACT—Objective:** Neutrophil extracellular traps (NETs) defend against acute infections. However, their overexpression causes organ failure during sepsis. Control of NET formation may improve the outcomes of patients with sepsis. Equol, a soybean isoflavone, is a female hormone analog, which prevents inflammation. We evaluated the effects of equol on NET formation in human neutrophils during inflammatory stimulation *in vitro*. **Methods:** Healthy volunteers provided blood samples. An enzyme-linked immunosorbent assay (ELISA) assessed serum equol concentrations. NET formation in neutrophils was induced by lipopolysaccharide (LPS) treatment. ELISA quantified DNA-binding elastase, and immunostaining assessed NET formation. Reverse-transcription quantitative PCR and western blotting detected G-protein-coupled receptor 30 (GPR30) or peptidyl arginine deiminase 4 (PAD4) expression. Flow cytometry assessed neutrophil phagocytic ability with inactivated *Escherichia coli*. **Results:** In neutrophils derived from males with low-serum equol levels (low-serum equol group), equol significantly decreased DNA-binding elastase levels and NET formation. Equol did not decrease NETs in neutrophils from males with high-serum equol levels. GPR30 expression of neutrophils was higher in the low-serum than in the high-serum equol group. PAD4 mRNA levels and nuclear PAD4 protein expression also decreased than the vehicle control in the low-serum equol group. Equol did not alter the

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3 phagocytic ability of neutrophils. In neutrophils from young females, equol had no  
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6 inhibitory effect on NET formation. **Conclusions:** Equol decreases LPS-induced NET  
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9 formation in neutrophils from males via inhibition of PAD4 expression. Our findings  
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12 provide a rationale for investigating a new therapeutic approach using equol to control  
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15 neutrophil activity during sepsis.  
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22 KEYWORDS—Female hormone analog; isoflavone; LPS; NETs; peptidyl arginine  
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## INTRODUCTION

Neutrophils play a crucial role in host defense during infection. The most well-known and important function of neutrophils is to accumulate at the site of infections and phagocytose the pathogens. New aspects of neutrophil function were revealed in 2004.<sup>1</sup> Neutrophil extracellular traps (NETs) are reticular structures released from neutrophils in response to stimuli. The NETs comprise DNA and histones from the cell nucleus and contain numerous intracytoplasmic granule components, such as elastases and myeloperoxidases. Upon encountering a stimulus, such as pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns, the levels of reactive oxygen species (ROS) in neutrophils increase and promote the activation of peptidylarginine deiminase (PAD) 4. PAD4 is an enzyme that catalyzes the conversion of arginine residues to citrulline in the presence of calcium ions.<sup>2</sup> Citrullination allows the decondensation of chromatin to create NETs. The NETs formed in the neutrophils are subsequently released into the extracellular space.<sup>3-6</sup> The formation of NETs is a key antimicrobial strategy employed by neutrophils.

During sepsis—a dysregulated immune response to an infection that leads to organ dysfunction—neutrophils release excessive NETs, which can cause endothelial and organ damage, further exacerbating inflammation.<sup>7,8</sup> Therefore, regulating the

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3 formation of NETs and their clearance could be an important therapeutic approach for  
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7 sepsis.

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10       There are sex-based differences in the prognosis of sepsis, with women having  
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12 a higher survival rate than men.<sup>9-11</sup> A reason for this difference is the anti-inflammatory  
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14 effect of female hormones. However, the potential benefit of administering female  
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16 hormones to improve survival during sepsis is still unknown. Moreover, considering its  
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18 side effects, such hormone therapy needs to be improved. Equol, [7-hydroxy-3-(49-  
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20 hydroxyphenyl)-chroman], is a metabolite derived from soybean isoflavone that is  
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22 structurally similar to the female hormone 17 $\beta$ -estradiol and has an affinity for estrogen  
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24 receptors (ERs).<sup>12,13</sup> In particular, S-equol has a stronger affinity for ER $\beta$ , which  
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26 mediates anti-inflammatory signaling.<sup>14,15</sup> It also binds to a membrane estrogen  
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28 receptor, G-protein-coupled receptor (GPR) 30, and leads to anti-inflammatory  
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30 signaling.<sup>16</sup> Because these estrogen receptors are present in neutrophils, we surmised  
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32 that equol could affect the formation of NETs. We hypothesized that equol could  
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34 decrease NET formation during inflammatory conditions, such as sepsis, and might  
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36 effectively treat male and postmenopausal female patients.  
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54       In this study, we demonstrate that equol can decrease the formation of NETs in  
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56 human neutrophils during acute inflammatory conditions *in vitro*. We chose  
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lipopolysaccharide (LPS), a typical PAMP, as a stimulator of NET formation. We examined the molecular mechanisms underlying NET formation to better understand its relationship with PAD4 expression.

## MATERIALS AND METHODS

### *Participants*

The study protocol was approved by the Ethics Committee of Hyogo Medical University (Approval No.: #3301). Ten healthy male (28–46-years old) and five healthy female (29–44-years old) volunteers were enrolled in this study from August 2019 to July 2023 after obtaining their informed consent.

### *Neutrophil isolation*

Neutrophils were isolated from whole blood using a neutrophil isolation kit (MACSxpress<sup>®</sup>: Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Anticoagulant-mixed whole blood was pipetted into a tube, a cocktail for magnetically labeling non-target cells was added, and it was incubated for 5 min at 20 °C. The tube was placed in the magnetic field of a MACSxpress<sup>®</sup> separator for 15 min. Labeled cells (lymphocytes and monocytes)



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3 adhered to the walls of the tubes, and erythrocytes precipitated at the bottom. The  
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6 neutrophil supernatant was carefully collected while the tube was inside the  
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10 MACSxpress separator. The isolated neutrophils were recovered by centrifugation at  
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13  $270 \times g$  for 10 min at 20 °C. Residual erythrocytes were removed by treatment with an  
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16 RBC lysis buffer at 20 °C for 10 min. The viability of cells was assessed using Trypan  
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19 Blue staining and was found to be >98%.  
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## 25 *Cell culture*

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28 Neutrophils (final concentration,  $1.0 \times 10^6$  cells/mL) were diluted in RPMI  
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31 1640 medium supplemented with 2% fetal bovine serum and 2.5 mM  $\text{CaCl}_2$  (NET  
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34 assay buffer) and plated on a collagen-coated 24-well plate (in the case of  
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37 immunofluorescence staining, collagen-coated 96-well plates were used). Neutrophils  
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41 were preincubated with LPS (LPS: E. coli O55:B5, Sigma-Aldrich, St. Louis, MO,  
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44 USA, final concentration 1  $\mu\text{g/mL}$ ) at 37 °C in the presence of 5%  $\text{CO}_2$  for 15 min.  
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47 After preincubation, S-Equol (Toronto Research Chemicals, Toronto, Canada) was  
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50 added to the neutrophils at a final concentration of 0.01–100  $\mu\text{M}$  and incubated at  
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53 37 °C in the presence of 5%  $\text{CO}_2$  for 30 to 165 min (total incubation time was 45–180  
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56 min, including preincubation). Dimethyl sulfoxide (DMSO  $\leq 0.002\%$ ; Sigma-Aldrich),  
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the solvent used for preparing the equol solution, was used as the vehicle control.

#### ***Assessment of serum equol concentration***

Serum equol concentrations were assessed using the Equol ELISA Kit (HelthecareSystems, Aichi, Japan) according to the manufacturer's instructions. Briefly, the serum and equol standards were incubated with an equol detection antibody at 22 °C for 1 h, then 3,3',5,5'-tetramethylbenzidine substrate solution was added and the mixture was further incubated at 22 °C for 15 min in the dark. After adding the stop solution, the absorbance was read at 450 nm using a microplate reader (SPECTRAmax PLUS384, Molecular Devices, San Jose, CA, USA) to assess the equol concentration. The detection limit of ELISA was 0.15 µM, and the lower limit of quantification was 0.72 µM. The upper limit of measurement was 103 µM.

#### ***Assessment of NETosis (DNA-binding elastase)***

After 3 h of neutrophil incubation, the levels of DNA-binding elastase were assessed using the NETosis Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. Briefly, plates were centrifuged at  $300 \times g$  for 3 min using a plate centrifuge (Model 2410, Kubota, Japan). The supernatant was

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3 gently aspirated from the wells to remove soluble neutrophil elastase not associated  
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6 with NETs, and the NET assay buffer was slowly added. The diluted S7 Nuclease Assay  
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9 Reagent was added to each well and incubated at 37 °C for 15 min to disrupt the NETs.  
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12 The supernatants were transferred to new microtubes, and ethylenediaminetetraacetic  
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15 acid (10 mM) was added to each tube to inactivate the nuclease. The mixture was  
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18 centrifuged at  $300 \times g$  for 5 min to remove cell debris. The supernatants and elastase  
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21 standards were incubated with an elastase substrate at 37 °C for 2 h, and the absorbance  
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24 was read at 405 nm using a microplate reader to assess the NETs formed.  
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### 32 *Immunofluorescence staining*

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35 After 3 h of incubation with equol, neutrophils were fixed with 4%  
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37 paraformaldehyde for 30 min at room temperature. The plates were centrifuged ( $300 \times g$ ,  
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39 2 min), and the supernatant was aspirated. The neutrophils and NETs were washed with  
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41 phosphate-buffered saline (PBS, pH 7.4) and incubated with an anti-histone H1 antibody  
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43 (ab71594 mouse monoclonal, Abcam, Cambridge, UK) and an anti-elastase antibody  
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45 (ab68672 rabbit polyclonal, Abcam) prepared in 1% bovine serum albumin in PBS at 4 °C  
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48 for 3 h. The neutrophils were washed with PBS and incubated with Alexa Fluor 488-  
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51 conjugated anti-rabbit IgG (Thermo Fisher Scientific Inc., Waltham, MA, USA) or  
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NorthernLights 557-conjugated anti-mouse IgG (R&D Systems Inc., Minneapolis, MN, USA) for 30 min at room temperature in the dark. After washing with PBS, the nuclei of the neutrophils were stained with 4',6-diamidino-2-phenylindole (Sigma-Aldrich). All the solutions added during the staining procedure were gently trickled down the wall of the plate, considering the fragile nature of NETs. The NETs were identified by their morphology and positive staining for histone H1 and neutrophil elastase using an inverted fluorescence microscope (ECLIPSE Ti-E, Nikon, Tokyo, Japan). The area occupied by the NETs was determined as the percentage of pixels showing NET-positive color in the field of view. NET-positive colors (red to yellow) were defined using hue, saturation, and brightness, and the percentage of pixels in the total field of view was calculated. Three wells/group were created, and photographs were taken for 26 fields/well ( $\times 100$  magnification) to cover the entire area of the well.

### ***Real-time reverse transcription PCR***

After 45 and 60 min from the start of the culture, total RNA was extracted using the TRIzol<sup>®</sup> reagent (Invitrogen, Carlsbad, CA, USA) and Direct-zol<sup>™</sup> RNA MicroPrep (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. An equal volume of ethanol was added to a sample lysed in the TRIzol reagent, and the

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3 solution was mixed thoroughly. It was then transferred to a Zymo-Spin IC column,  
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6 placed in a collection tube, and centrifuged at  $15,000 \times g$  for 30 s. The flow-through  
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9 was discarded, and the column was placed in a new tube. After adding DNase I to the  
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12 column to degrade any contaminating DNA, the Direct-zol RNA PreWash buffer was  
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15 added and centrifuged at  $15,000 \times g$  for 30 s. The flow-through was discarded, and the  
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18 washing step was repeated. The RNA wash buffer was added to the column and  
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21 centrifuged at  $15,000 \times g$  for 1 min. The column was transferred to a new RNase-free  
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24 tube, and DNase/RNase-free water was added to the column, followed by centrifugation  
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27 at  $15,000 \times g$  for 30 s. The cDNA was synthesized using the iScript cDNA Synthesis Kit  
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30 (Bio-Rad) according to the manufacturer's instructions. The following cDNAs were  
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33 then quantified using quantitative real-time RT-PCR analysis with SYBR Green  
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36 Realtime PCR Master Mix (Toyobo, Osaka, Japan) and primers for the PAD4 gene  
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39 (*padi4*; F: 5'-accagctgtgaaagatcaga-3', R: 5'-tcacagttcaccagcaggat-3') on a Light  
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42 Cyclar 480 II (Roche Diagnostics, Basel, Switzerland). Relative expression of each  
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45 cDNA was calculated using the ddCt method after normalizing against peptidylprolyl  
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48 isomerase A gene (*ppia*; F: 5'-atctgcactgccaagactgag-3', R: 5'-gaaggaatgatctggtggttaaga-  
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51 3') expression. We used the Human Housekeeping Gene Primer Set (Takara Bio Inc.,  
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54 Shiga, Japan) and assessed the data using the BestKeeper software<sup>17</sup> to choose the  
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appropriate internal control. The ppia gene showed the most stable expression in the study samples.

### ***Western blotting***

At 45, 120, and 180 min from starting the culture, cytoplasmic and nuclear proteins were extracted from the neutrophil pellet using the LysoPure Nuclear and Cytoplasmic Extractor kit (Fujifilm Wako Chemical, Osaka, Japan) according to the manufacturer's instructions. Briefly, neutrophils were collected in 1.5 mL microcentrifuge tubes and centrifuged at  $200 \times g$  for 5 min at 4 °C. The supernatant was discarded, and the neutrophils were resuspended in 1 mL PBS and transferred into a new prechilled microcentrifuge tube. The tubes were centrifuged again, and the supernatant was aspirated. Nuclear Fraction Buffer (100  $\mu$ L) was added to the pellet, and the tubes were vortexed. After 10 min of incubation on ice, the tubes were centrifuged at  $500 \times g$  for 10 min at 4 °C. The cytoplasmic protein supernatant was transferred, and the tubes were centrifuged at  $20,000 \times g$  for 10 min at 4 °C. The pellets were washed with 500  $\mu$ L of Nuclear Fraction Buffer, and 50  $\mu$ L of Nuclear Extraction Buffer was added. The tubes were incubated on ice for 30 min and vortexed every 10 min. The tubes were centrifuged at  $20,000 \times g$  for 10 min at 4 °C, and then the

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3 supernatant containing the nuclear proteins was transferred into a new prechilled  
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6 microcentrifuge tube. The protein concentration was measured using DC protein assay  
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9 reagents (Bio-Rad Laboratories, Hercules, CA, USA). The protein concentration was  
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12 adjusted to the concentration compatible with an automated western blot system and  
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15 stored at  $-80^{\circ}\text{C}$  until measurement.  
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19 The Abby™ Simple Western automated nano-immunoassay system  
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22 (ProteinSimple, San Jose, CA, USA), a capillary-based system for size separation of  
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25 proteins, was used to determine the protein expression. Samples were processed  
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28 according to the manufacturer's standard method for the 12–230 kDa Abby separation  
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31 module (SM-W004). Briefly, samples were mixed with fluorescent molecular weight  
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34 markers and 400 mM dithiothreitol (Ez standard Pack 1, ProteinSimple Inc., Santa  
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37 Clara, CA, USA) at a final concentration of  $0.25\text{ }\mu\text{g}/\mu\text{L}$  and then denatured at  $95^{\circ}\text{C}$  for  
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40 5 min. The migration of proteins through the separation matrix was performed at 375 V  
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43 for both samples and ladder (12–230-kDa, PS-ST01EZ, ProteinSimple Inc.). The  
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46 separated proteins were immobilized using the photoactivated capture chemistry within  
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49 ProteinSimple Inc.'s proprietary. Subsequently, samples were incubated with primary  
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52 antibody (rabbit polyclonal anti-GPR30 antibody, bs1380, Bioss, Boston, MA, USA or  
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55 rabbit polyclonal anti-PAD4 antibody, ab96758, Abcam) for 30 min followed by a wash  
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step and 30 min incubation with an anti-rabbit secondary antibody (DM-001, ProteinSimple Inc.). Peroxide/luminol-S (DM-001, ProteinSimple Inc.) was used for chemiluminescent detection, and the Total Protein Detection Module (DM-TP01, ProteinSimple Inc.) was used for normalization by total protein. The Compass Simple Western software (version 6.0.1, ProteinSimple Inc.) was used for the automatic calculation of the heights (chemiluminescence intensity), area, and signal/noise ratio, as well as for capturing the digital image of the capillary chemiluminescence. Electrophoretic patterns of total protein confirmed the absence of nuclear and cytoplasmic protein contamination.

### ***Flow cytometry***

Neutrophils ( $1.0 \times 10^6$  cells/mL) were diluted in the NET assay buffer and preincubated with LPS in 1.5 mL tubes at 37 °C in the presence of 5% CO<sub>2</sub> for 15 min. After preincubation, equol (0.01–0.1 μM) or DMSO (≤0.002%) was added to the neutrophil suspension and incubated at 37 °C in the presence of 5% CO<sub>2</sub> for 60 min. At 75 min after the start of culture, pHrodo Green *E. coli* BioParticles Conjugate for Phagocytosis (Thermo Fisher Scientific) was added into the wells, and the plates were incubated at 37 °C in the presence of 5% CO<sub>2</sub> for 15 min. The neutrophils were



collected in FACS tubes, and the tubes were centrifuged at  $350 \times g$  for 5 min. After discarding the supernatant, cell pellets were resuspended in 500  $\mu$ L of PBS. In the flow cytometry analysis (FACSCant II, Becton Dickinson, San Jose, CA, USA), the neutrophil population was gated using forward and side scatter. The area positive for phagocytosis was defined using *E. coli*-free neutrophils as a negative control.

### ***Statistical analysis***

Data are shown as mean  $\pm$  standard deviation. The normality of the data distribution was verified using the Shapiro–Wilk test. Paired *t*-test was used when a parametric test was appropriate, and Wilcoxon signed-rank test was used when a non-parametric test was warranted. All tests were two-sided, and a *p* value  $< 0.05$  was considered statistically significant. All statistical analyses were performed using JMP software version 15.2 (SAS Institute, Cary, NC).

## **RESULTS**

### ***Equol decreased the levels of DNA-binding elastase in LPS-treated human neutrophils***

We examined the effect of equol on neutrophils from male volunteers, which

are less affected by female hormones. First, we measured the serum equol concentrations of volunteers. Among the ten serum samples, in only one (subject j), the serum equol concentration (1.38  $\mu$ M) exceeded the lower limit of quantitation of 0.72  $\mu$ M. The remaining nine samples (subjects a-i) had concentrations above the detection limit but below the quantitation limit, with a mean concentration of 0.50  $\mu$ M (characterized as a low serum equol group) (Fig.1 A).

Next, we checked the release of DNA-binding neutrophil elastase from LPS-treated neutrophils obtained from male volunteers using NETosis ELISA.

In the low serum equol group, equol at the lower concentrations (0.01 and 0.1  $\mu$ M) significantly decreased the amount of DNA-binding neutrophil elastase compared with DMSO (vehicle control) (Fig. 1B). At higher concentrations (1–10  $\mu$ M), equol exhibited a tendency to decrease the amount of DNA-binding neutrophil elastase compared to DMSO (Fig. 1B). Neutrophils obtained from subject j, who had high serum equol concentrations, had lower amounts of DNA-binding elastase. The addition of 0.01  $\mu$ M equol decreased DNA-binding elastase, while at 1  $\mu$ M, it showed a tendency to increase (Fig.1C).

### ***Effect of GPR30 expression on neutrophils***

We investigated whether the addition of equol affects GPR30 expression in neutrophils.

First, we examined GPR30 expression in neutrophils belonging to the low serum equol group. Figure 2A represents a typical blot for GPR30 expression. The GPR30 expression in each sample was quantified by normalizing it against total protein levels (Fig. 2B). Neutrophil GPR30 expression was observed in all samples, and the addition of equol did not change the expression levels (Fig. 3E serum equol low group).

Next, we examined GPR30 expression in neutrophils from subject j, who had higher serum equol concentrations. Given that ELISA showed that the neutrophils of subject j tended to increase NET levels when exposed to high concentrations (1  $\mu$ M) of equol, we further confirmed the expression of GPR30 with the addition of higher concentrations of equol (100  $\mu$ M). Neutrophils of subject j exhibited minimal expression of GPR30 across all concentrations, suggesting that the addition of equol had little effect on neutrophils via GPR30 in the subject j (Figs. 2C, D, and E).

#### ***Area occupied by NETs in LPS-treated neutrophils was decreased by equol.***

Fluorescent immunostaining was performed to morphologically confirm the effect of equol on the area occupied by NETs in LPS-treated neutrophils. Figure 3A

shows images of typical NET formation in DMSO- and equol-treated groups at three different concentrations. The area occupied by NETs was greater in DMSO treatment than in equol treatment. Similar to the ELISA results, 0.01 and 0.1  $\mu$ M equol significantly decreased, and 1  $\mu$ M equol solution tended to reduce the area occupied by NETs (Fig. 3B).

#### ***Equol decreased the expression of PAD4 mRNA***

To determine the effect of equol on PAD4 expression, we measured the mRNA levels in neutrophils, which belonging to the low serum equol group in male, treated with equol. Because the effective concentration of equol, as determined using ELISA and immunofluorescence staining, was 0.01  $\mu$ M, we used the same concentration for this experiment and the ones that followed. At 45 min after the start of culture (i.e., 30 min after the addition of equol), the expression of PAD4 mRNA was significantly decreased in the equol treatment than in the DMSO treatment (Fig. 4). At 60 min after the start of culture (i.e., 45 min after the addition of equol), the expression of PAD4 mRNA tended to be lower in the equol treatment than in the DMSO treatment, but the difference was not significant (Fig. 4).

***Equol decreased the nuclear PAD4 expression at 45 min after LPS treatment.***

To determine the nuclear transfer of PAD4, we measured the cytoplasmic and nuclear expression of PAD4 using western blotting. Figure 5A represents a typical blot for cytoplasmic PAD4 expression. The PAD4 expression in each sample was quantified by normalizing it against total protein levels (Fig. 5B). The cytoplasmic PAD4 expression showed no differences between the equol and DMSO treatments at any of the time points (Fig. 5C). Figures 5D and E show typical images for nuclear PAD4 expression and total protein expression, respectively. At 45 min, PAD4 expression in the equol treatment was lower than that in the DMSO treatment (Fig. 5D), whereas the total protein in the equol treatment was higher than that in the DMSO treatment (Fig. 5E). The typical PAD4 band in the equol treatment indicated significantly lower expression than that in the DMSO treatment at 120 and 180 min (Fig. 5D); however, the total protein levels were also lower in these lanes (Fig. 5E). The mean of the results for the five male volunteers showed that equol significantly decreased the nuclear expression of PAD4 than DMSO only at 45 min (Fig. 5F).

***Equol did not alter the phagocytic ability of neutrophils***

To determine whether equol affected the phagocytic ability of neutrophils, we

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3 evaluated the uptake rate of fluorescently labeled inactivated *E. coli* by neutrophils  
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6 using flow cytometry and considered it to reflect the phagocytic ability of neutrophils.  
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9 Neutrophils without fluorescently labeled, inactivated *E. coli* were used as negative  
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12 controls.  
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16 Equol showed a similar uptake ratio (phagocyte-positive ratio) compared with  
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18 DMSO at each concentration. There were no significant differences in the phagocytic  
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21 ability of the DMSO and equol treatments (Fig. 6).  
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28 ***Inhibitory effect of equol on NET formation was lower in neutrophils from female***  
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31 ***volunteers***  
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35 We tested whether equol could decrease the formation of NETs in neutrophils  
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37 from female volunteers. First, we checked the serum equol concentrations in the female  
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40 volunteers. In three of the five serum samples (subjects m, n, and o), the concentration  
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43 exceeded the lower limit of quantitation of 0.72  $\mu\text{M}$ . The mean concentration was 1.06  
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46  $\mu\text{M}$ . For the remaining two samples (subjects k and l), the concentration was above the  
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49 detection limit but below the quantitation limit (Fig.7 A). The mean concentration was  
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53 0.35  $\mu\text{M}$ .  
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57 Figure 7B shows the average area occupied by NETs in subjects m, n, and o,  
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3 who exhibited higher concentrations of serum equol. The area occupied by NETs  
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6 showed no significant decrease in the treatment with equol (Fig. 7B). The images of the  
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9 NET formation in subject l are shown in Figure 7C. Similar NET formation was  
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12 observed in the treatments with DMSO and equol at concentrations of 0.01 to 100  $\mu$ M.  
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15 Figure 7D shows the average area occupied by NETs in subjects k and l. The  
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18 morphological measurement of NETs showed no changes, even at 10 and 100  $\mu$ M of  
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21 equol (Fig. 7D).  
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## 28 **DISCUSSION**

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32 This is the first study to demonstrate that equol can decrease LPS-induced  
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35 formation of NETs via inhibition of the PAD4 pathway in human neutrophils. At lower  
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38 concentrations (0.01 to 0.1  $\mu$ M), equol significantly decreased the formation of NETs.  
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41 Still, the higher concentrations (1 to 10  $\mu$ M) of equol tended to reduce the formation of  
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44 NETs in neutrophils obtained from male volunteers who showed low serum equol levels.  
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47 Furthermore, equol decreased the nuclear expression of the PAD4 protein and its mRNA.  
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50 Because the PAD4 pathway is associated with organ dysfunction in sepsis,<sup>18,19</sup> the PAD4  
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53 inhibitory effect of equol may improve the outcome in male patients with sepsis. In  
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56 addition, because the phagocytic ability was not altered by equol treatment, equol may be  
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3 able to suppress the formation of NETs and reduce septic organ failure without  
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6 compromising infection defense. In contrast, equol failed to reduce the formation of NETs  
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9 in neutrophils obtained from female volunteers, regardless of serum equol concentrations.  
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12 The inhibitory effect of equol on NET formation may have some relation with biological  
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15 sex.  
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19 Equol is a substance produced from daidzein, a soy isoflavone. It is metabolized  
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22 by bacteria in the intestines. It has all the benefits of isoflavones; for example, it exhibits  
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25 estrogen-like, antiandrogenic, and antioxidant effects.<sup>20</sup> Intestinal bacteria metabolize  
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28 isoflavones. Hence, in the absence of certain intestinal bacteria, isoflavones are  
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30  
31 ineffective.<sup>21</sup> However, if an isoflavone is administered in the form of equol, no further  
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34 metabolizing is needed, and the desired benefits can be obtained even if the patient does  
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37 not have the required intestinal bacteria. Our results show that equol could suppress the  
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40 excessive formation of NETs and prevent organ damage via the suppression of PAD4 in  
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43 males, who may have fewer anti-inflammatory effects of female hormones. Suppression  
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46 of neutrophil NETs is effective in preventing organ damage; however, from the  
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49 perspective of defense against infections, transient suppression of excessive NETs with  
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52 rapidly metabolizing agents, rather than their long-term suppression, is important to  
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55 improve patient outcomes. Oral administration of equol results in a rapid increase in its  
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3 blood levels, followed by its quick excretion. The plasma elimination half-life of equol is  
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6 approximately 8 h.<sup>22</sup> Although studies on equol have only focused on its oral  
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9 administration,<sup>23,24</sup> considering its use in emergency and intensive care settings, we  
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12 believe that exploring intravenous administration may be a worthy avenue to investigate  
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15 the transient suppression of NETs, depending on the patient's condition.  
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19 Individuals lacking equol-producing bacteria cannot produce equol even if they  
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22 ingest soy isoflavone. The population of equol producers was reported to be 50–70% in  
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25 East Asian countries, whereas it was 20–30% in Western countries.<sup>25–28</sup> Blood levels of  
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28 equol differ significantly between equol producers and non-producers. For instance,  
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31 Zhang et al. reported that the blood equol concentration in 40–49-year-old Japanese men  
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34 was 0.055  $\mu\text{M}$  (interquartile ranges (IQR): 0.025–0.138  $\mu\text{M}$ ,  $n = 125$ ) in equol producers  
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37 and 0.0001  $\mu\text{M}$  (IQR: 0.0001–0.005  $\mu\text{M}$ ,  $n = 177$ ) in non-producers.<sup>29</sup> Equol production  
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40 is considerably influenced by diet; among the Japanese population, older people produce  
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43 more equol because of their consumption of traditional soybean-rich products. Sekikawa  
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46 et al. reported the serum concentration of equol in Japanese patients aged 75–89 years ( $n$   
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49 = 91, male/female, 45/46); the average concentration of equol in 45 patients was not  
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52 detectable (0  $\mu\text{M}$ ), 22.8  $\mu\text{M}$  (IQR; 9.2–31.4  $\mu\text{M}$ ) in 23 patients, and 166.1  $\mu\text{M}$  (IQR;  
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55 109.0–256.4  $\mu\text{M}$ ) in the 23 patients.<sup>30</sup> In our study, the serum concentration of equol was  
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0.18  $\mu$ M to 1.38  $\mu$ M in 15 volunteers (male 10 and female 5 volunteers). These concentrations were higher than those of equol-producers previously reported by Zhang et al. but lower than those of equol-low producers previously reported by Sekikawa et al. It could not be determined from the previous report whether our volunteers were equol-producers or not. In this experiment, the concentration of serum equol above 0.71  $\mu$ M, which was the lower limit of quantification of the ELISA, was defined as the high equol group and those below it as the low equol group. In male subjects, equol near blood concentrations (0.01-0.1  $\mu$ M) suppressed NET formation, while equol above blood concentrations (1-10  $\mu$ M) had a less pronounced suppressive effect on NETs. Owing to the presence of limited amounts of neutrophils *in vitro* conditions, the ER $\beta$  and GPR30 binding of equol may have reached a plateau; therefore, the inhibitory effect on NETs was not significant at the higher concentrations. In addition, because DMSO showed a trend toward suppression of NETs at higher concentrations, making it difficult to determine the difference. The suppressive effect of equol on NETs was not pronounced in samples with high blood levels of equol but low expression of GPR30. Given that the addition of high concentrations of equol did not increase receptor expression, the effect of equol may depend on both the blood concentration of equol and the individual differences in the original receptor expression level. However, we think that the high concentration of equol

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3 may potentially inhibit NET formation. Pazourekova et al. reported that a high  
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6 concentration of equol suppressed ROS production in neutrophils.<sup>31</sup> The inhibition of  
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9 ROS production leads to PAD4 nuclear transfer inhibition, which may decrease NET  
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12 formation. However, their experiments have not been effective in reducing ROS  
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15 production at concentrations that were effective in our experiments. Different pathways  
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18 may be postulated for the inhibitory effect of equol on NETs, depending on its  
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21 concentration.  
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26       There are several types of NETs, such as suicidal or vital NETs. Suicidal NETosis  
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28 depends on ROS activation, which leads to histone citrullination by PAD4 and  
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30 PKC/mitogen-activated protein kinases pathways, and occurs within 2–4 h.<sup>32</sup> Vital  
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32 NETosis, which occurs without the loss of the nuclear or plasma membrane, occurs within  
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35 5–60 min and is independent of ROS activation.<sup>33–35</sup> DNA and cytoplasmic granules are  
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38 released through a small area in the neutrophil cell surface during vital NETosis, allowing  
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41 the neutrophils to continue phagocytosing pathogens.<sup>36</sup> Because our results showed NET  
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44 formation at 3 h after LPS stimulation, we believe equol could decrease suicidal NETosis.  
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48 The results of PAD4 also showed that equol could decrease the formation of suicidal  
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51 NETs. Equol suppressed NET formation but did not increase phagocytosis. As such, there  
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54 is skepticism about equol directly affecting vital NETs or increasing the function of  
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3 neutrophils that evade NETs. A detailed report revealed time-dependent changes in  
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6 phorbol myristate acetate (PMA)-stimulated NET formation in neutrophils *in*  
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9 *vitro*.<sup>3</sup> According to this report, ~60 min after PMA stimulation was “the no-return point”  
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12 for chromatin swelling, and irreversible suicidal NETs were formed.<sup>3</sup> At 45 min, but not  
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15 at 60 min, after LPS stimulation, the expression of PAD4 mRNA was significantly  
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18 decreased by equol. The nuclear translocation of the PAD4 was also significantly  
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21 inhibited at 45 min after LPS stimulation of neutrophils. The presence or absence of equol  
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24 does not appear to affect the expression of PAD4 mRNA or nuclear translocation of the  
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27 protein after 60 min of NET-inducing stimulation. Because NET formation was  
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30 suppressed by equol at 3 h, we surmised that adding equol within 60 min of the NET-  
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33 inducing stimulus might be important. Equol was added 15 min after LPS stimulation,  
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36 which indicates that even this late addition of equol is effective in producing the desired  
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39 results.  
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44 In neutrophils obtained from female volunteers, equol had no inhibitory effect  
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47 on NETs. Molero et al. suggested that the expression of ER $\alpha$  and ER $\beta$  in human  
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50 neutrophils depends on plasma estrogen concentrations.<sup>37</sup> As higher estrogen  
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53 concentrations cause higher receptor expression, it is possible that women express more  
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56 ER $\alpha$  and ER $\beta$  than men. In our results, adding equol did not increase GPR30 in samples  
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3 obtained from men. In neutrophils of female origin, the expression of GPR30 may not be  
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6 much different from that of males. We are examining GPR30 expression in female  
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9 subjects and have not found significant differences from men (data not shown). Further  
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12 investigation is needed to determine the details, which will be the subject of future  
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15 research. Equol does not have an inhibitory effect on NETs in neutrophils of female origin,  
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18 which may be related to the fact that female hormones increase ER $\beta$  and ER $\alpha$ . Equol has  
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21 a higher affinity for ER $\beta$  and GPR30 than ER $\alpha$ ; however, it can bind ER $\alpha$ .<sup>12-15</sup> At equol  
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24 concentrations higher than those under physiological conditions or when neutrophils  
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27 express high levels of ER $\alpha$ , the ratio of binding to ER $\alpha$  may be increased. ER $\alpha$  has been  
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30 reported to induce the expression of inflammatory factors in neutrophils and neutrophil  
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33 activation.<sup>38</sup> This can be favorable for the host because it leads to early pathogen  
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36 elimination. In our experimental conditions, ER $\alpha$ -derived inflammatory and ER $\beta$ -derived  
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39 anti-inflammatory effects antagonized each other. They did not sufficiently suppress the  
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42 formation of NETs in female samples and higher equol concentrations in male samples.  
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47 In addition to hormones, there have been reports of genetic differences in immune  
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50 response; therefore, genetic differences in neutrophils between sexes may also be  
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53 involved.<sup>39</sup> Because we hypothesized that administering equol to patients with low female  
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56 hormone levels would be effective, we did not examine optimal neutrophil concentrations  
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3 derived from volunteers with high female hormone levels. However, we believe that high  
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6 concentrations of equol may be effective in neutrophils with high levels of female  
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9 hormones, which is worth further investigation. This suggests that physiological  
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12 concentrations of equol may suppress NET formation. In other words, using “extremely  
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15 high” concentrations of equol may not be necessary for patients with sepsis.  
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19 The limitation of this study was that we did not measure blood levels of female  
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22 hormones; therefore, the relationship between equol and female hormones remains to be  
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25 determined. The female volunteers were of the fertile generation; we therefore do not  
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28 know the effect on postmenopausal women. In addition, because we did not measure the  
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31 expression of the estrogen receptors in neutrophils, we could not determine the  
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34 relationship between the ERs and the effective concentration of equol. *In vivo*  
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37 experiments should be considered in the future to further investigate the effective  
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40 concentration of equol.  
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45 Our study provides a rationale for investigating a new therapeutic approach using  
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48 equol to control neutrophil function in sepsis. Equol is a stable substance that can be  
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51 produced inexpensively, and if it can alleviate sepsis exacerbations, it could contribute to  
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54 reducing healthcare costs.  
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## Figure legends

### **FIG. 1. Quantitation of serum equol or DNA-binding neutrophil elastase using**

**ELISA.** (A) Serum equol concentration of male volunteers. The a-j symbols indicate

individual subjects. The dashed line indicates the limit of quantitation for the ELISA.

Measurements were performed in duplicate for each subject. Data show mean  $\pm$  standard

deviation (SD). (B) Quantifying DNA-binding neutrophil elastases in the serum equol

low group (subjects a-i). Data show mean  $\pm$  SD values for 10 (0.01  $\mu$ M), 10 (0.1  $\mu$ M), 11

(1  $\mu$ M), and 4 (10  $\mu$ M) volunteers. Measurements were performed in duplicate for each

subject. \*\*,  $p < 0.01$ , as determined using the Wilcoxon signed rank test. (C) Quantifying

DNA-binding neutrophil elastases in a serum equol high volunteer (subjects j).

Measurements were performed in duplicate for each subject. Data show mean  $\pm$  SD.

### **FIG. 2. GPR30 expression of human neutrophils.** (A) A typical western blotting result

for the serum equol low group. GPR30 was detected as a band of approximately 52 kDa.

(B) The electrophoresis patterns of the total protein used for western blotting are

described in panel A. (C) A typical western blotting result for subject j. GPR30 was

detected as a band of approximately 52 kDa. (D) The electrophoresis patterns of the total

protein used for western blotting are described in panel C. (E) The average ratio of GPR30 protein expression. Data show mean  $\pm$  SD values ( $N = 7$ ; serum equol low group).

**FIG. 3. Immunofluorescence staining of neutrophil extracellular traps in neutrophils from male volunteers.** (A) Typical images were obtained using immunofluorescence staining. Cells were stained for histone H1 (red), elastase (green), and DNA (blue): scale bars, 200  $\mu$ m. Photographs were taken at 100 $\times$  magnification. (B) The average ratio of NET-positive color pixels to the total pixels in the field of vision. Data show mean  $\pm$  SD values for neutrophils from four volunteers ( $N = 5$ ). Measurements were performed in triplicate for each subject. \*\*,  $p < 0.01$ , as determined using the Wilcoxon signed rank test.

**FIG. 4. Expression of PAD4 mRNA in male serum of equol low group at 45 and 60 min after the start of incubation.**

The average PAD4 mRNA levels are represented. The vertical axis shows expression relative to that at 45 min after the start of incubation in the DMSO group. Measurements were performed in duplicate for each subject. Data show mean  $\pm$  SD values ( $N = 5$ ). \*\*,  $p < 0.01$ , as determined using the paired  $t$ -test.

**FIG. 5. Expression of PAD4 protein in the male serum equol low group at 45, 120, and 180 min after the start of incubation.** (A) A typical western blotting result for cytoplasmic proteins. PAD4 was detected as a band of approximately 74 kDa. (B) The electrophoresis patterns of the total protein used for western blotting are described in panel A. Cytoplasmic protein showed many bands of approximately 40–66 kDa in size. (C) The average ratio of PAD4 protein expression in the cytoplasmic fraction. Data show mean  $\pm$  SD values ( $N = 5$ ). The data were statistically analyzed using paired *t*-tests. Measurements were performed in duplicate for each subject. (D) A typical western blotting result for nuclear proteins. PAD4 was detected as a band of approximately 74 kDa. (E) The electrophoresis patterns of the total protein used for western blotting are described in panel D. Nuclear protein showed numerous bands of various sizes, with the most intense band detected between 66 and 116 kDa. (F) The average ratio of PAD4 protein expression in the nuclear fraction. Data show mean  $\pm$  SD values ( $N = 5$ ). Measurements were performed in duplicate for each subject. \*,  $p < 0.05$ , as determined using the paired *t*-test or Wilcoxon signed rank test.

**FIG. 6. Equol treatment did not alter the phagocytic ability of neutrophils.** The average intake levels of fluorescently labeled inactivated *Escherichia coli* were measured



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3 using flow cytometry. The vertical axis shows the phagocyte-positive ratio, which  
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6 indicates the percentage of neutrophils that have taken up fluorescently labeled dead  
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9 bacteria. Measurements were performed in duplicate for each subject. Data show mean  $\pm$   
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13 SD values ( $N = 3$ ).  
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19 **FIG. 7. Quantitation of DNA-binding elastase and neutrophil extracellular trap**  
20 **(NET) formation in neutrophils obtained from female volunteers.** (A) Serum equol  
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22 concentration of female volunteers. The k-o symbols indicate individual subjects. The  
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24 dashed line indicates the limit of quantitation for the ELISA. Measurements were  
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26 performed in duplicate for each subject. Data show mean  $\pm$  standard deviation (SD). (B)  
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28 The average ratio of NET-positive color pixels to the total pixels in the field of vision in  
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30 subjects m, n, and o. Data show mean  $\pm$  SD values ( $N = 3$ ). Measurements were performed  
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32 in triplicate for each subject. (C) Typical images were obtained using  
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34 immunofluorescence staining (subject l). Cells were stained for histone H1 (red), elastase  
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36 (green), and DNA (blue): scale bars, 200  $\mu$ m. Photographs were taken at  $\times 100$   
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38 magnification. (D) The average ratio of NET-positive color pixels to the total pixels in  
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40 the field of vision in subjects k and l. Data show mean  $\pm$  SD values ( $N = 2$ ). Measurements  
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42 were performed in triplicate for each subject.  
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Fig.1

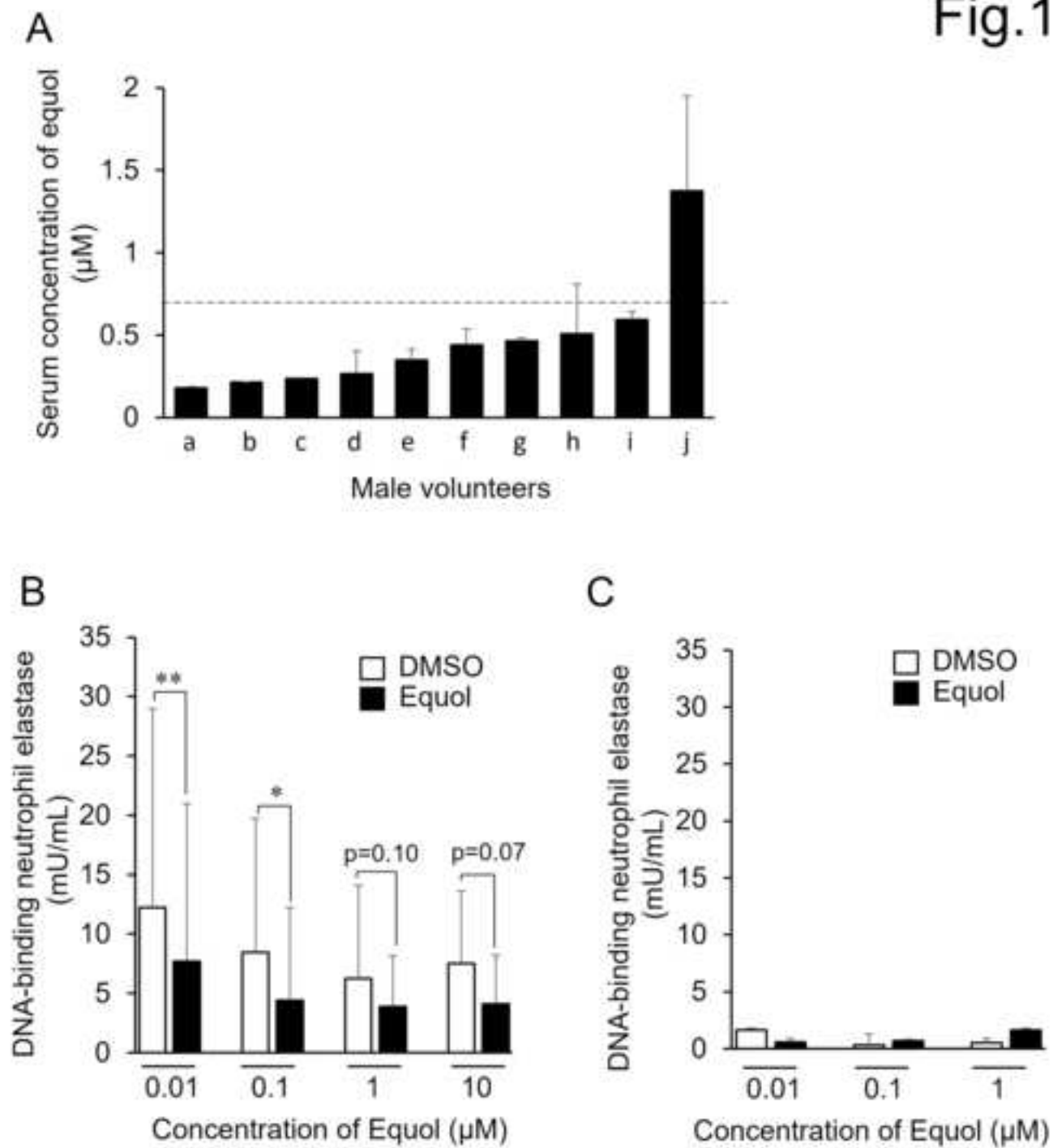
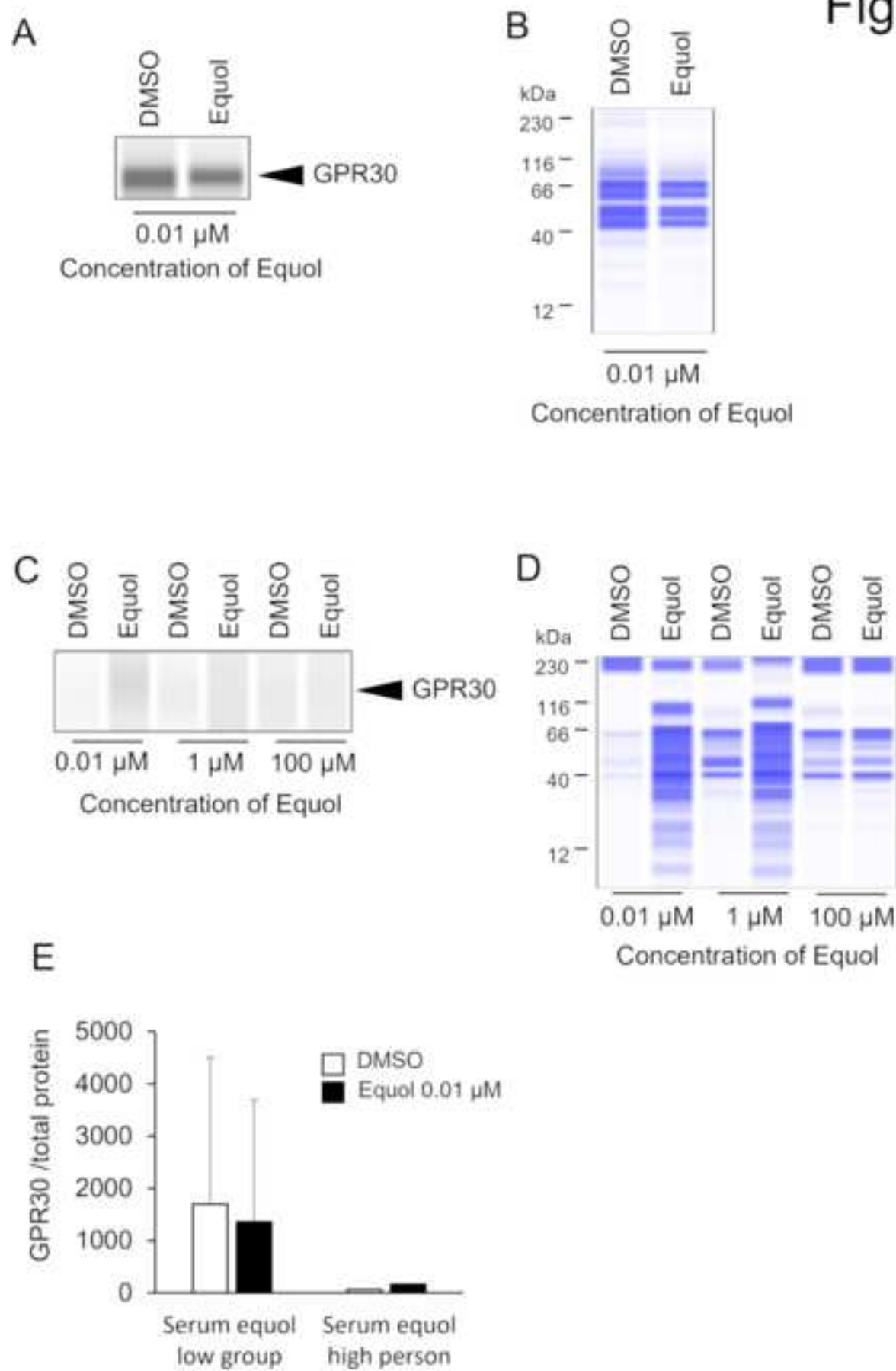


Fig.2



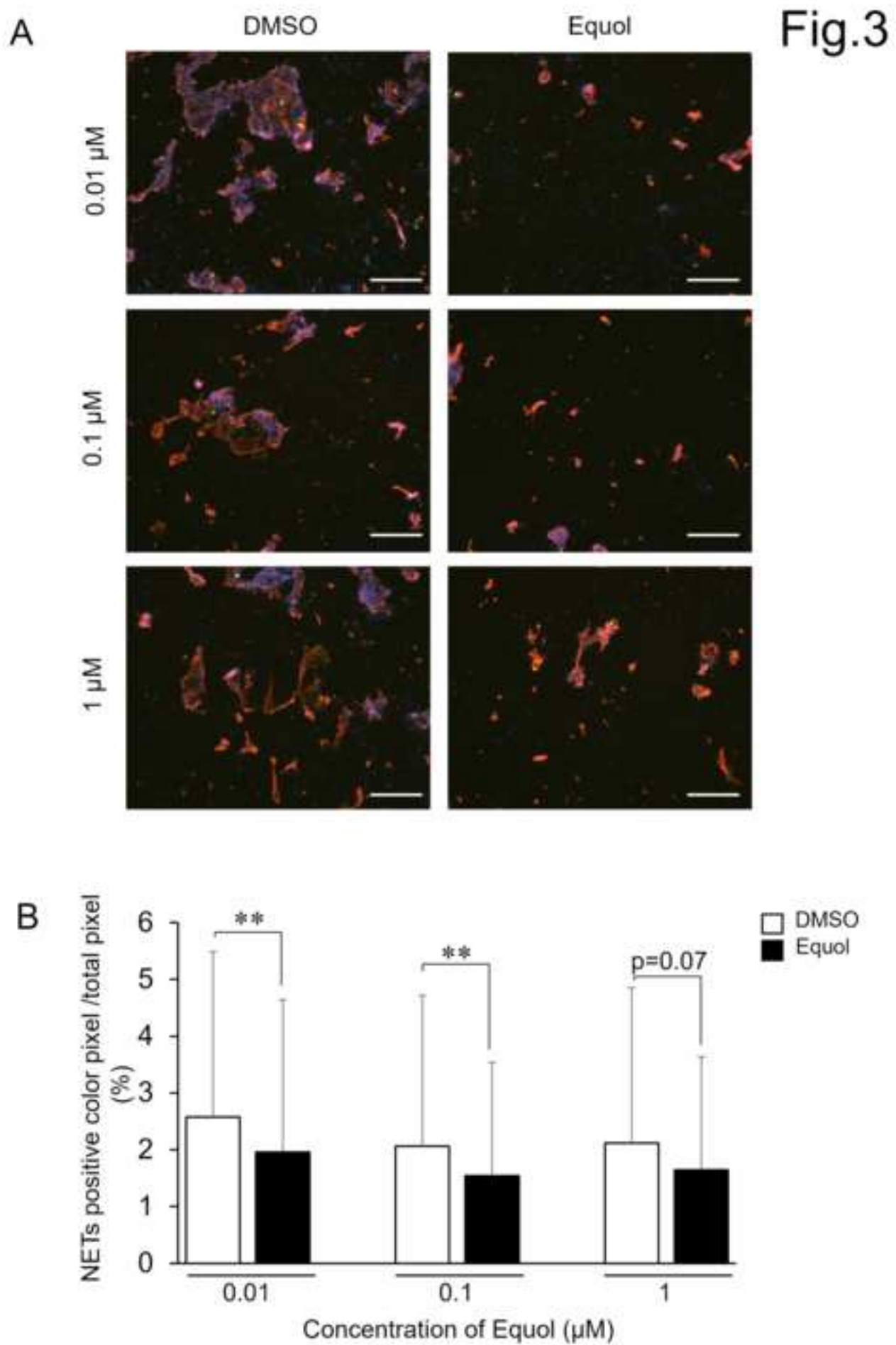


Fig.4

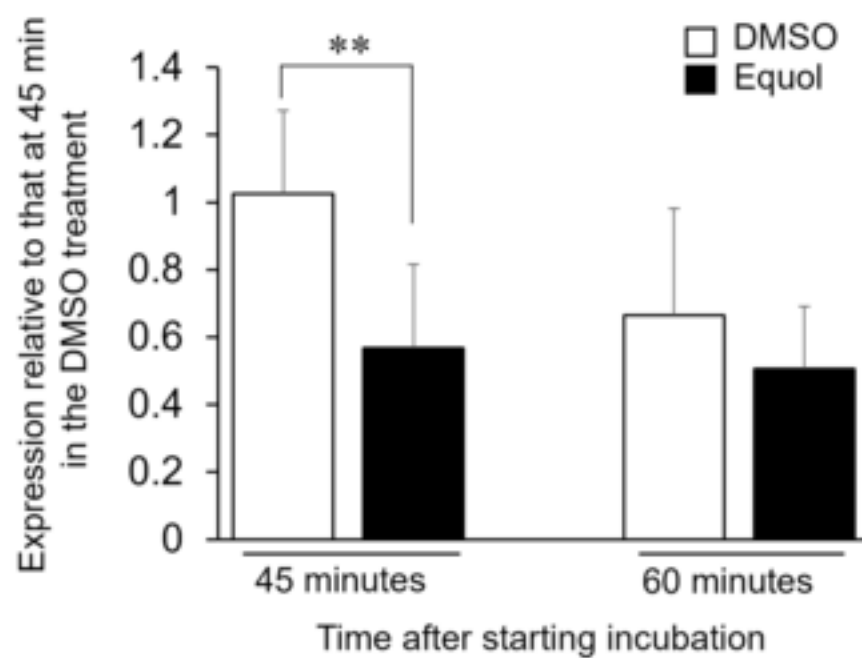


Fig.5

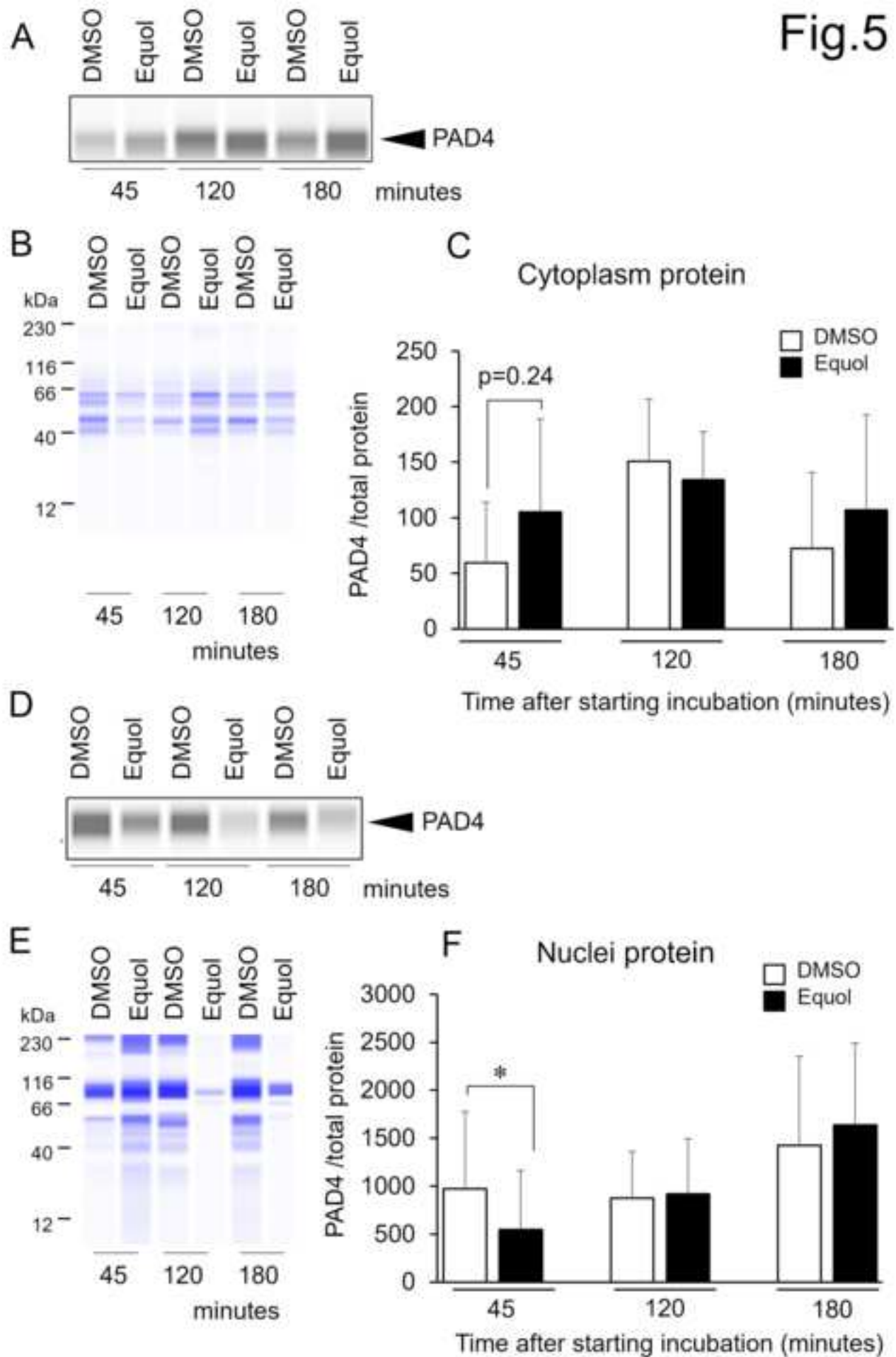


Fig.6

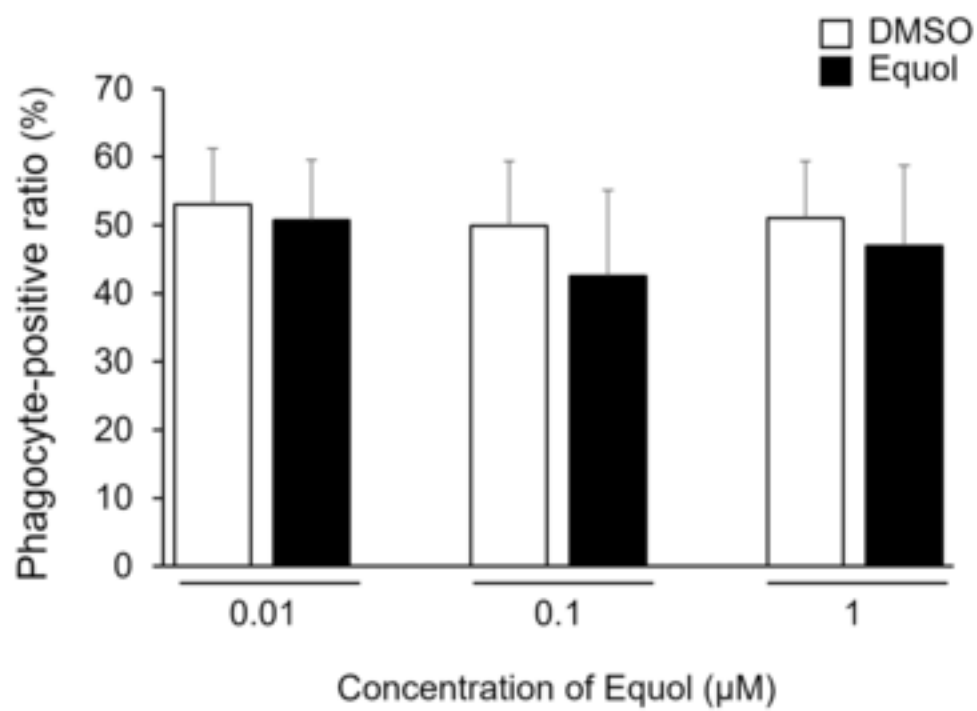


Fig.7

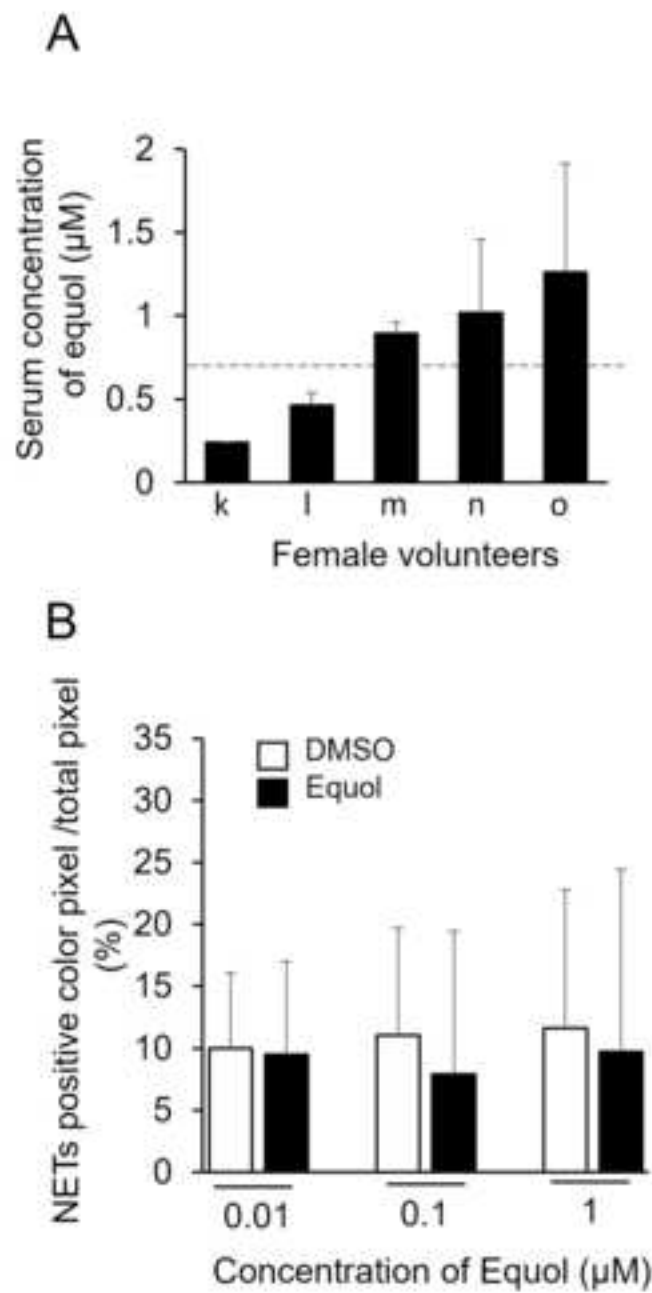




Fig.7

