

Flavonoids baicalein and kaempferol reduced inflammation in benign prostate hyperplasia patient-derived cells through regulating mitochondrial respiration and intracellular oxygen species

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Abstract Benign prostate hyperplasia (BPH) is one of the most common elderly disease, and because of prolonged incubation period and many side effects of medication or surgical interventions, the use of dietary phytochemicals is considered as an effective measure for prevention of BPH. The purpose of this study is to investigate the mechanism of inhibition effect for BPH by flavonoids such as baicalein and kaempferol. BPH cells were collected through biopsy from patients with PSA of 4 or higher, followed by primary culture. In vitro experiments were conducted to evaluate mitochondrial respiration, intracellular reactive oxygen species (ROS) level and expression of inflammatory markers, genes, and anti-oxidants. In conclusion, baicalein and kaempferol have been demonstrated to inhibit BPH through lowering ROS, thereby reducing inflammation triggers, and reduced inflammation. This study is expected to be helpful in the development of flavonoids that have a clinical effect on suppressing BPH.

Keywords: flavonoid, BPH, mitochondria, ROS, inflammation

Introduction

Benign prostatic hyperplasia (BPH) is one of the most common urological diseases in elderly people, characterized by low mortality and high morbidity, and is considered as a public health problem (Isaacs, 1994). Symptoms caused by prostatic hyperplasia are various and cause anxiety, sleep disturbances, decreased mobility, and difficulty in sexual intercourse, leading to working and social activities disturbance and negatively affect the quality of life (Bosch et al., 1995; Kirby, 2000). Because of the high incidence, prolonged incubation period of BPH and many side effects of medication or surgical interventions, the use of dietary phytochemicals is considered as an effective measure for prevent BPH (Dedhia and McVary, 2008; Fourcade et al., 2008).

Various types of plant extracts are commercially available, and representatively, there is saw palmetto (*Serenoa repens*) bean extract, and the brand names are Saw Palmetto in South Korea and Permixon in Europe. The mechanisms of inhibition of prostatic hypertrophy of *Serenoa repens* extract (SRE) is known as anti-androgen effects, anti-inflammatory effects, and pro-apoptotic properties, but it has not been clearly defined and did not show a significant difference from placebo in one study (Keehn and Lowe, 2015).

Flavonoids are a type of secondary metabolite of plants or fungi classified as anthoxanthin, flavanone, flavanonol, flavan and anthocyanidin. Kaempferol and baicalein are kinds of flavonoids and are well known for their anti-inflammatory properties (Devi et al., 2015; Lee et al., 2015).

It is generally accepted that intracellular reactive oxygen species (ROS), generated from mitochondrial respiration, is associated with inflammation and increase in cell growth factors, which can lead to prostate cancer or prostatic hyperplasia (Ahmad et al., 2012; Aydin et al., 2006; Bostanci et al., 2013; De Nunzio et al., 2011; Kullisaar et al., 2012; Sciarra et al., 2008). Inflammatory markers such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) are associated with BPH induction (Abdel-Aziz et al., 2020). Nuclear factor erythroid-2(NFE2)-related factor 2 (Nrf2)-Kelch-like ECH-associated protein 1 (Keap1) pathway is an important regulator in protecting cells against oxidative stress. They bind to the antioxidant response element and activate the transcription of major antioxidant enzymes (Kansanen et al., 2013; Lee et al., 2013).

The purpose of this study is to determine whether the anti-inflammatory action of flavonoids, kaempferol and baicalein, can inhibit BPH through detection of mitochondrial respiration, intracellular ROS level, anti-oxidants such as iNOS, COX-2, Nrf2, and Keap1. Identification of the in vitro mechanism of flavonoids can be used as basic data for future clinical applications.

Materials and Methods

Collection of human prostate specimen

Prostate tissue obtained from prostate biopsy samples performed on patients at Severance hospital, South Korea. Prostate biopsy

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was performed on patients with a prostate specific antigen (PSA) of 4.0 or higher, and only patients with a pathologic report of benign prostatic hyperplasia were involved. The Institutional Review Board of Severance Hospital approved this study protocol (Approval No: 4-2017-0300). Informed consent was waived by the board.

Primary cell culture

Primary hyperplastic prostate cells were obtained by cutting the prostate tissue finely, treating it with collagenase and trypsin, and incubating for 5-10 minutes at 37°C. Primary cells were cultured using Dulbecco Modified Eagle Medium (DMEM). A complex medium with 10% (w/v) FBS (Fetal Bovine Serum) and 1% (w/v) penicillin streptomycin added to each medium was prepared and used for cultivation. 37°C, 5% CO₂ incubator (Forma 3546, ThermoScientific, Inc., Massachusetts, USA) was used in the experiment while subculturing at intervals of 4 days.

Serenoa repens extract preparation

The stock solution of *Serenoa repens* extract (SRE) was prepared as an ethanolic (20% solution, wt/vol) extract of Saw Palmetto Berry Powder (PharmaPrint, Irvine, CA, USA) with constant agitation for at least 10 hours at room temperature. The solution was filtered through 3- μ m filter and centrifuged at 1000 g for 10 min to remove debris. The extract then concentrated by passing a stream of nitrogen over it and was stored in the dark at room temperature until use (Goldmann et al., 2001).

Measurement of the cellular energy consumption through mitochondrial respiration

Kaempferol and baicalein are purchased from Sigma-Aldrich (St. Louis, MO, USA). After treatment with 200 nl of SRE and 10 μ M of baicalein or kaempferol, measurement of the mitochondrial respiration of primary prostate cells using Agilent Seahorse Xfp (Agilent Technologies, Santa Clara, CA, USA) was processed. Cells cultured with DMEM were treated with materials and cultured for 24 hours. The SRE group was used as positive control group and the untreated group was used as the control group in this experiment. Recovered cells were transferred to an XFP assay plate. The concentration of the cells was maintained at 1×10^4 cells/well, and cultured for 1 hour in a non-CO₂ incubator with DMEM medium supplemented with 4.5 g/L D-glucose, L-glutamine, and sodium pyruvate. At this time, the temperature was set to 37°C, and the medium was maintained at pH 7.4. Cells were sequentially treated with mitochondrial inhibitors oligomycin (1.0 μ M), Trifluoromethoxy carbonyl cyanide phenylhydrazine (FCCP) (1.0 μ M), and rotenone/antimycin A (0.5 μ M) according to the manufacturer's protocol. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), indexes of oxidative phosphorylation, were calculated with Seahorse™ XFP analyzer and software (Agilent Technologies).

Intracellular reactive oxygen species (ROS) level measurement

Intracellular ROS level was evaluated using 2',7'-dichloro-

fluorescein diacetate (H₂DCFDA; Invitrogen, USA). H₂DCFDA is deacetylated by intracellular esterase to form H₂DCF, which is oxidized by ROS to strong fluorescent compounds, 2',7'-dichloro-fluorescein (DCF). Dichlorofluorescein diacetate (DCFDA; Sigma, USA) was used as a fluorescent probe to measure the change in the concentration of active oxygen in cells. DCFDA, a non-fluorescent substance, is oxidized by ROS in the presence of peroxides related to hydrogenperoxide in the cell, resulting in green fluorescence. Therefore, the measurement of ROS can be made through DCFDA. 2×10^{-5} primary hyperplastic prostate cells were inoculated into a 60 mm culture dish and cultured for 24 h. After treatment with the sample and H₂O₂, it was cultured for 2 h. Before harvesting the cells, 10 μ M DCFDA was treated in the medium and cultured for 30 min. Cultured cells were washed with phosphate-buffered saline (PBS) and treated with 1% trypsin-EDTA solution to harvest the cells, washed again with PBS to resuspend the harvested cells, and in preheated PBS containing 10 μ M DCFDA for 30 minutes at 37°C. After incubation, it was then transferred to a 5 mL FACS tube (SPL Life Science, Pocheon, Korea). Subsequently, the mean fluorescent intensity (MFI) was determined for the 10,000 cells of each sample using a FACS Calibur flow cytometer (Becton Dickinson, NJ, USA) at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Data analysis was performed using Cell Quest software (USA).

Anti-inflammatory gene and protein expression

Observe the expression of iNOS, COX-2, Nrf2, and Keap1 of primary prostate cell groups treated with flavonoids, SRE (positive control) and control untreated group through Western Blotting (Mahmood and Yang, 2012). After being incubated for 24 hours treated with flavonoids, primary prostate cells were collected and were lysed in 60 μ L of NP40 (Invitrogen, USA) containing protease inhibitors and then incubated on ice for 30 to 40 min. Proteins were measured using bicinchoninic acid (BCA) protein assay kits (Thermo Fisher Scientific, USA) and separated by polyacetylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Germany). The membranes were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline containing 0.5% tween-20 (T/TBS) for 1 h at room temperature. After blocking, incubation with 1:1000 dilution of primary antibodies for 24 h at 4°C occurred, using iNOS antibody (1:5000, Calbiochem, USA), COX-2 antibody (1:1000, BD Biosciences Pharmingen, USA), or β -actin antibody (1:10000, Sigma, USA). The membranes were washed four times in T/TBS for 15 min and incubated with secondary antibodies for 1 h, room temperature. After washing three times with T/TBS, separated protein bands were detected with an ImageQuant LSA4000 (GE Healthcare, USA) using ECL solution (Dyne, Korea).

Real-time polymerase chain reaction (RT-PCR) analysis was done to evaluate Nrf2 mRNA level. Primary prostate cells treated with flavonoids were homogenize and separated by centrifugation for 15 minutes at 4°C. The mRNA was isolated using RNeasy columns (Bio-Rad, Hercules, CA, USA) and the cDNA was

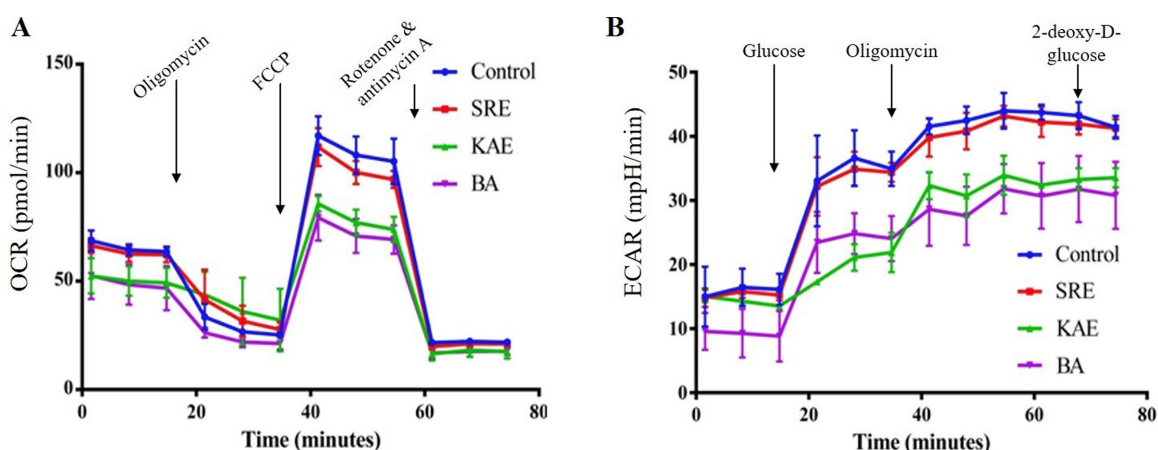


Fig. 1. Cellular oxygen consumption. (A) Oxygen consumption rate (OCR) and (B) Extracellular acidification rate (ECAR) of hyperplastic prostatic cells treated with flavonoids. Data represent the mean±standard error (n=3); OCR, Oxygen consumption rate; FCCP, Trifluoromethoxy carbonyl cyanide phenylhydrazine; ECAR, Extracellular acidification rate; SRE, *Serenoa repens* extract; KAE, Kaempferol; BA, Baicalein

synthesized using a high-capacity cDNA reverse transcriptase kit (Applied Biosystems, Life Technologies, Foster City, CA, USA). RT-PCR was performed using a CFX Real-Time PCR System (Bio-Rad).

Results and Discussion

Cellular mitochondrial respiration

OCR and ECAR using oligomycin, FCCP, rotenone and antimycin-A were measured to analyze the change in oxygen consumption more specifically (Zhang and Zhang, 2019). Oligomycin, ATP synthase inhibitor in the electron transport chain (ETC), is added following the basal measurement to decrease OCR, resulting a reduction in mitochondrial respiration or OCR. Using the difference between basal respiration and oligomycin-stimulated reduced OCR, the oxygen consumption for ATP synthesis can be calculated (Lee et al., 2019; TeSlaa and Teitell, 2014). Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazine (FCCP) collapses the proton gradient, making the electron flow unaffected by ETC. Oxygen consumption by complex IV in ETC is maximized (Lee et al., 2019).

Rotenone and antimycin-A act to shutdown electron transporting systems by inhibiting mitochondrial complexes I and III. Therefore, Using FCCP-stimulated OCRs, the spare respiratory capacity defined as the difference between maximum and basal respiration can be calculated. Spare respiratory capacity refers to the ability of cells to respond to stress or increase their energy demand (Lee et al., 2019).

The OCR and ECAR results are illustrated in Fig. 1. The mitochondrial respiration of each sample and control group was measured. In the basal respiration measured before oligomycin was added, it showed that the basal respiration of the sample group was weaker than that of the control and SRE groups. The maximal respiration measured after FCCP addition was also decreased in the sample group compared to the control and SRE groups. In addition, low ECAR after glucose treatment was observed compared to the control and positive control SRE group (Fig. 1).

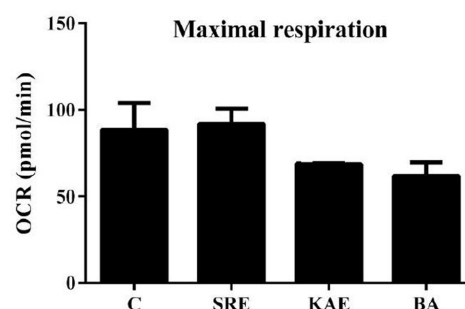


Fig. 2. Cellular mitochondrial maximal respiration. The maximal mitochondrial respiration was calculated based on OCR. Untreated group was used as control. Data represent the mean±standard error (n=3); OCR, Oxygen consumption rate; C, Control group; SRE, *Serenoa repens* extract; KAE, Kaempferol; BA, Baicalein

In both baicalein and kaempferol treated group, spare respiratory, difference between maximal respiration and basal respiration, were decreased compared with control and SRE groups, since the sample groups showed a remarkable decrease in maximal respiration compared to basal respiration. No significant difference was observed between baicalein and kaempferol (Figs. 1 and 2). It has been demonstrated that flavonoid treatment caused a decrease in cellular respiration, and it can be inferred that this will lead to a decrease in ROS.

Intracellular ROS level

The generation of intracellular ROS showed through DCF-fluorescence and flow cytometric distribution of DCFDA-stained primary hyperplastic prostate cells were presented in Fig. 3. It was determined whether the intracellular ROS level actually decreased in the group treated with baicalein and kaempferol, and the baicalein-treated group showed 40% reduction in ROS concentration compared to the control group without any treatment, and about 60% in kaempferol. These experimental results are consistent with the experimental results in which mitochondrial cell respiration, which is related to the production of active oxygen in cells,

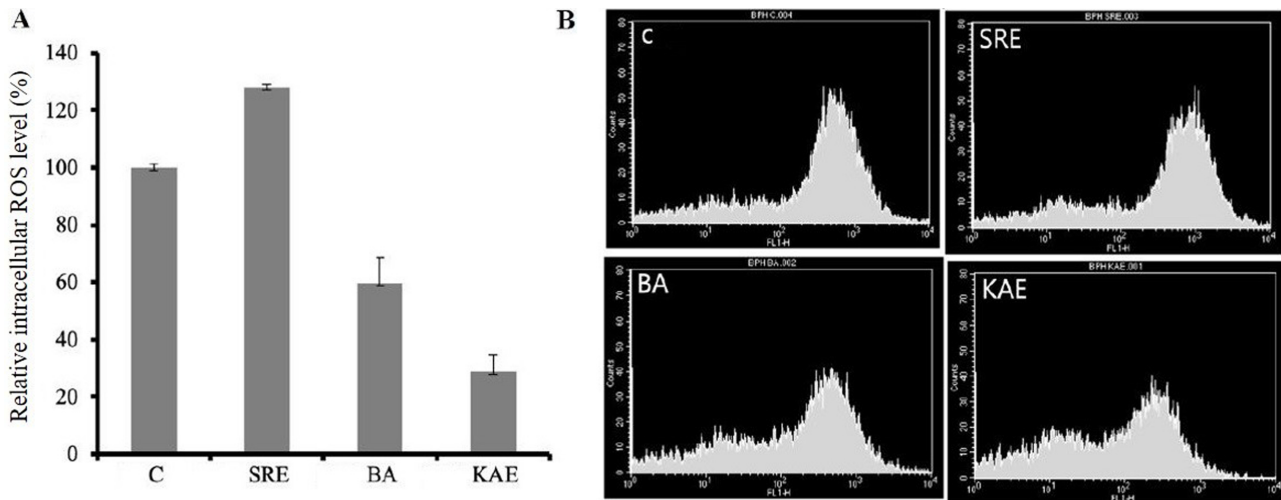


Fig. 3. Intracellular reactive oxygen species level. Intracellular ROS level of primary hyperplastic prostate cells treated with flavonoids was measured using H2DCFDA. (A) Relative intracellular ROS compared to control group. The control group was set to 100 and the MFI values for each group were calculated as relative values. (B) Flow cytometric distribution of DCFDA-stained primary hyperplastic prostate cells. Untreated group was used as control. Data represent the mean±standard error (n=3); ROS, reactive oxygen species; BPH, Benign prostatic hyperplasia; C, Control group; SRE, *Serenoa repens* extract; KAE, Kaempferol; BA, Baicalein

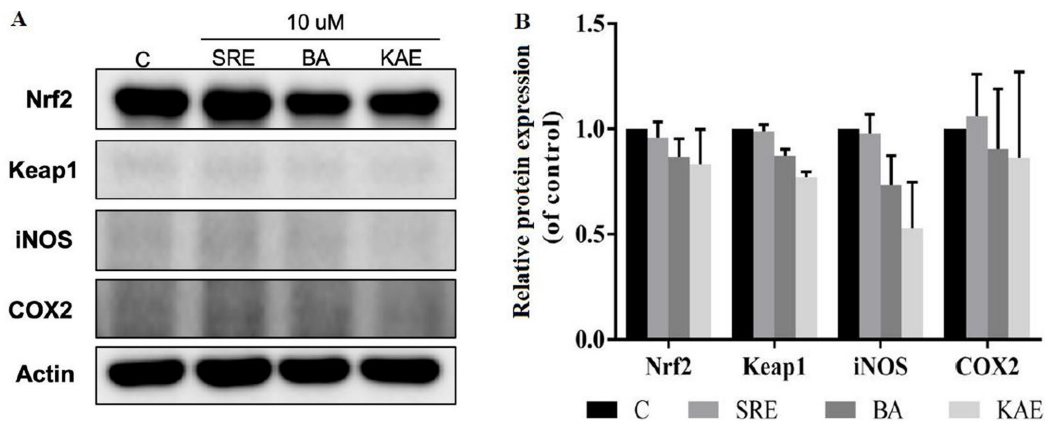


Fig. 4. Expression of anti-oxidants and inflammatory markers. (A) Western blotting analysis for protein expression. (B) Densitometry of repeated western blotting three times. Untreated group was used as control. Data represent the mean±standard error; C, Control group; SRE, *Serenoa repens* extract; KAE, Kaempferol; BA, Baicalein; Nrf2, nuclear factor erythroid-2-related factor 2; Keap1, Kelch-like ECH-associated protein 1; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2

decreased (Figs. 1~3). Through this, it could be inferred that baicalein and kaempferol would have the effect of preventing BPH through the mechanism of reducing the intracellular ROS level.

Anti-inflammatory gene and anti-oxidant expression

In order to elucidate the mechanism of reducing these inflammatory factors, the expression of the inflammatory factors iNOS and COX-2, and the antioxidant enzyme regulator Nrf2 was observed. iNOS and COX-2 are attracting attention as being associated with BPH induction (Abdel-Aziz et al., 2020).

When the expression level of the untreated control group was set to 1, the expression levels of nuclear factor erythroid-2(NFE2)-related factor 2 (Nrf2), Kelch-like ECH-associated protein 1 (Keap1), iNOS, and COX-2 in the positive control SRE group were measured as 0.96, 1.07, 0.98, 1.06, and the baicalein group was 0.87, 0.99, 0.73, 0.91, and the kaempferol group was

measured as 0.83, 0.83, 0.53, 0.86, in the same order (Fig. 4). Nrf2 expression at mRNA level was slightly increased in the group treated with baicalein and SRE, and showed decrease slightly in the group treated with kaempferol (Fig. 5).

Both iNOS and COX-2 decreased in the baicalein and kaempferol groups, and there was no significant difference between the control and SRE groups, especially in the kaempferol-treated group, iNOS decreased by 45% compared to the control group. The expression of Nrf2 was not found to be related to the expression level of Keap1 (Fig. 4). Degradation of Keap1 releases Nrf2 bound to keap1 and moves to the nucleus of the cell, resulting in the expression of the downstream antioxidant protein of Nrf2 (Lee et al., 2013). The increasing in the amount of Nrf2 expression at the mRNA level can be understood as a mechanism to maintain the amount of Nrf2 in the cell due to the decrease in the total amount of Nrf2 (Fig. 5).

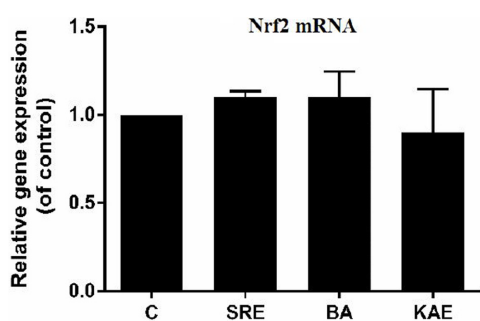


Fig. 5. Nrf2 mRNA expression. RT-PCR results for Nrf2 mRNA of hyperplastic prostatic cells treated with flavonoids. Untreated group was used as control. Data represent the mean \pm standard error; C, Control group; SRE, *Serenoa repens* extract; KAE, Kaempferol; BA, Baicalein; Nrf2, nuclear factor erythroid-2-related factor 2

As baicalein and kaempferol showed significant activity against primary prostate cells, the mitochondrial respiration volume, intracellular ROS concentration, and protein level expression of major inflammatory and antioxidant transcription factors were observed. It was confirmed that baicalein and kaempferol induce a decrease in mitochondrial respiration and free radicals in cells, thereby reducing the expression of iNOS and COX-2. This study is considered to be clinically useful because primary culture was performed with cells from patients with BPH rather than normal healthy prostate cells. However, this study has some limitations. At first, overall statistical significance was not secured. Second, this experiment is conducted *in vitro*, there is a question whether it will be applied equally to the actual human body. Although Prostate cells from BPH patients were used, but further research is needed for actual clinical use. Additional experiments with mice or rats are planned, and according to the results, experiments in humans are being planned.

In conclusion, flavonoids such as baicalein and kaempferol have been demonstrated to inhibit BPH through following mechanism: Flavonoids lower ROS, thereby reducing inflammation triggers, and reduced inflammation inhibits BPH. This study is expected to be helpful in the development of flavonoids that have a clinical effect on suppressing BPH.

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Conflict of Interest

The authors declare no conflict of interest.

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