A THESIS FOR THE DEGREE OF MASTER OF SCIENCE IN FOOD AND NUTRITION

Effects of apigenin and luteolin on p53-dependent apoptosis and autophagy in colon cancer cells

대장암세포에서 아피제닌과 루테올린이 p53 의존적 세포사멸과 자가포식에 미치는 영향

February, 2019

Department of Food and Nutrition

Graduate School

Seoul National University

Ho Soo Yoo

Abstract

Effects of apigenin and luteolin on p53-dependent apoptosis and autophagy in colon cancer cells

Ho Soo Yoo
Department of Food and Nutrition
Graduate School
Seoul National University

The flavonoids apigenin and luteolin exhibit anticancer effects in various types of cancer by the regulation of apoptosis and cell cycle arrest. Recently, autophagy was reported as one of the anticancer mechanisms of flavonoids. It was found that apigenin induces autophagy; however, the mechanisms of apigenin-induced autophagy and the regulation of autophagy by luteolin are unknown. The present study investigated whether apigenin and luteolin exhibit their anticancer effects by the induction of apoptosis and autophagy in a p53-dependent manner in colon cancer cells. To achieve this aim, colon cancer cell lines HCT-116 and HT-29 and colon fibroblast CCD-18Co were used. Also, the expression of related indicators in protein and mRNA levels was examined through western blot analysis and polymerase chain reaction (PCR) analysis. Apigenin at 12.5-25 μmol/L and luteolin at 10-20 μmol/L induced cytotoxicity

in p53 wild type HCT-116 colon cancer cells but not in p53 mutant HT-29 cells and normal colon cells. Apigenin at 1, 5 μmol/L induced cytotoxicity in p53 mutant HT-29 cells. Although low concentration of apigenin just inhibited cell cycle arrest in a p53 independent manner, the anticancer effect of apigenin and luteolin was exhibited as an increase in p53 phosphorylation and the expression of p53 downstream genes leading to apoptosis and cell cycle arrest in HCT-116 cells. The induction of endoplasmic reticulum stress was also observed in response to apigenin and luteolin in a p53-dependent manner. Finally, we showed that apigenin and luteolin can induce autophagy in p53 wild type cells but not in p53 mutant cells, which suggests that apigenin- or luteolin-induced autophagy is p53-dependent; however, the chloroquine (CQ)- mediated inhibition of autophagy did not upregulate the apoptosis of cells treated with apigenin or luteolin. In conclusion, the present data showed that apigenin and luteolin could inhibit the growth of colon cancer cells through p53-dependent regulation of apoptosis and autophagy.

Keywords: apoptosis, autophagy, colon cancer, apigenin, luteolin, p53

Student number: 2017-22435

Contents

Abstract……..i

Contents…….....iii

List of Tables…….....v

List of Figures……...vi

List of Abbreviations……..viii

I. Introduction……...…….1

II. Materials and Methods…….…9

1. Cell culture and treatment……9

2. Assessment of cell viability by trypan blue exclusion assay……...10

3. Total RNA extraction and quantitative real-time PCR analysis…..10

4. Semi-quantitative RT-PCR……...…12

5. Total protein extraction and western blotting analysis……..13

6. Measurement of autophagic flux…….……14

7. Statistical analysis……14

III. Results……...…15

1. Apigenin and luteolin reduced viability of colon cancer cells HCT-116 and HT-29, but not in normal colon cells CCD-18CO……..15

2. Apigenin and luteolin activated p53 by phosphorylation…….17

3. Luteolin and high concentration of apigenin induced p53-dependent apoptosis and cell cycle arrest…….…….…….19

4. Low concentration of apigenin induced p53 independent cell cycle arrest……23

5. Apigenin and luteolin increased ER stress in a p53 dependent manne..25

6. Apigenin and luteolin induced p53-dependent autophagy……..27
IV. Discussion……..32
V. References……...37
국문 초록……42

List of Tables

Table 1. The sequences of primer for real time PCR ……11

Table 2. The sequences of primer for semi-quantitative PCR.….....12

List of Figures

Figure 1. The process of apoptosis…….2

Figure 2. The process of autophagy…….4

Figure 3. The structure of apigenin and luteolin……...8

Figure 4. The cytotoxicity effect of apigenin and luteolin on cancerous cells HCT-116, HT-29, and non-cancerous cells CCD-18Co……16

Figure 5. The expression of phosphorylated p53 protein in HCT-116 and HT-29 treated with luteolin and high concentration of apigenin……..18

Figure 6. the expression of p53 activation indicator …….20

Figure 7. The expression of apoptosis indicator in HCT-116 and HT-29 treated with luteolin and high concentration of apigenin……21

Figure 8. The expression of cell cycle arrest indicator in HCT-116 and HT-29 treated with luteolin and high concentration of apigenin…….22

Figure 9. The expression of apoptosis and cell cycle arrest indicator in HCT-116 and HT-29 treated with low concentration of apigenin…….24

Figure 10. Apigenin and luteolin induced ER stress in p53 wild-type colon cancer cells……26

Figure 11. Apigenin and luteolin increased the protein expression of LC3-II in colon cancer cells in a p53-dependent manner……...28

Figure 12. Apigenin and luteolin increased the protein expression of p62 which is a marker autophagosome - lysosome fusion in colon cancer cells……29

Figure 13. Increase of LC3-II was not caused by inhibition of fusion with autophagosome and lysosome…….30

Figure 14. Autophagy inhibition by chloroquine did not affect apigenin and

luteolin-induced apoptosis and viability reduction in colon cancer cells……31

List of Abbreviations

AMPK: AMP-activated protein kinase

Atg: Autophagy related gene

ER: Endoplasmic reticulum

LC3: Microtubule-associated protein light chain 3

mTOR: Mammalian target of rapamycin

PCNA: Anti-proliferation cell nuclear antigen

PI3K: Phosphatidylinositol 3-kinase

I. Introduction

Colon cancer is one of the most common and fatal diseases in the world. Approximately 140,000 cases of colon cancer are diagnosed and 50,000 Americans are estimated to die from colon cancer annually in the United States. (Siegel et al. 2018). The global burden of colorectal cancer is expected to gradually increase. Similarly, a significant increase in the incidence and mortality of colorectal cancer was observed in 1983–2014 in Korea (Jung et al. 2017). Cancer is the most common cause of death, and it is the third most common cancer after stomach and liver cancers in Korea (Jung et al. 2017). The prevention and treatment of colon cancer is therefore becoming increasingly important in public health.

Apoptosis and autophagy turn over cytoplasmic organelles and entire cells, respectively (Marino et al. 2014). Because apoptosis and autophagy conduct basic processes in cells, we can consider that they determine the destiny of cells, i.e. their death or survival. From this point of view, the regulation of apoptosis and autophagy is related to anticancer effects.

Apoptosis is one of the various types of programmed cell death and is caused by the reception of intracellular and intercellular signals. The mechanism is commonly divided into intrinsic and extrinsic pathways **(Figure 1)**. The intrinsic pathway is initiated by signals that occur inside cells, such as the level of cytosolic $Ca^{2+}$and oxidative stress. These signals increase mitochondria permeability and release apoptotic factors, including cytochrome c. The pathway is regulated by the Bcl-2 family (e.g., Bax, Bad, Bak, Bcl-2, Bcl-xl, and Mcl-l) by blocking or promoting the release of cytochrome c. In the

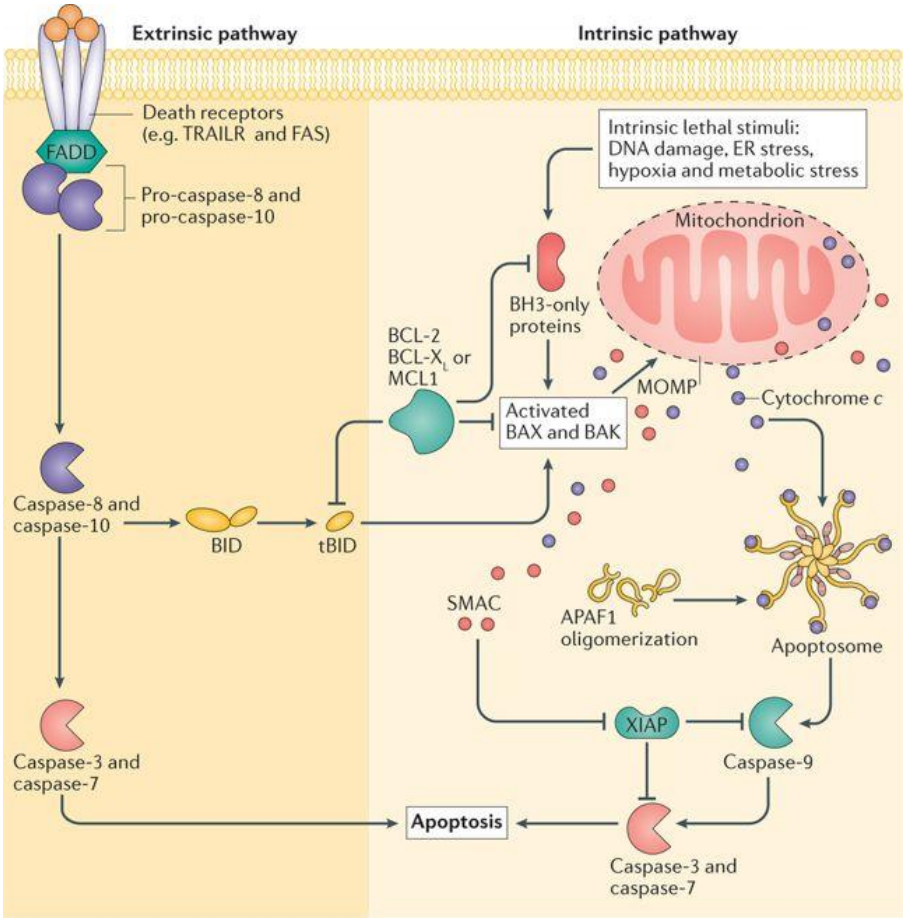

Figure 1. The process of apoptosis (Ichim et al. 2016).

TRAILR, TNF-related apoptosis-inducing ligand receptor; BID, BH3 interacting-domain death agonist; BCL, B-cell lymphoma 2; MCL, myeloid cell leukemia; BAK, Bcl-2 homologous antagonist/killer; MOMP, mitochondria outer membrane permeability; APAF, apoptotic protease activating factor 1; XIAP, X-linked inhibitor of apoptosis protein.

cytoplasm, cytochrome c activates caspases including caspase 3, 9 and then builds up apoptosome by combination with apf-1, cytochrome c, and caspase 9.

The extrinsic pathway is initiated by signals that occur outside cells, including death ligand TNF and Fas. When death ligands bind to death receptors on the plasma membrane, caspase initiator caspase 8 is activated by signal cascade inside cells (Wong 2011). Morphological hallmarks are nuclear fragmentation and condensation, plasma membrane blebbing, and apoptotic bodies (Marino et al. 2014). Because cells undergo the process of death through apoptosis, this mechanism is called a "self-killing system" (Maiuri et al. 2007).

Autophagy, which is called a "self-eating system," is a pivotal component of cellular adaptation in response to stress to maintain mammalian homeostasis (Maiuri et al. 2007). This is accomplished by the degradation of cellular components to maintain energy metabolism and to eliminate damaged cellular proteins and subcellular organelles, such as dysfunctional mitochondria under stress circumstances. Therefore, autophagy has been shown to protect against several diseases, including neurodegenerative and inflammatory diseases and cancer. In contrast, an impaired autophagy leads to cell death, chronic inflammation, and genetic instability (White et al. 2010).

There are three types of autophagy—macroautophagy, microautophagy, and chaperone-mediated autophagy; however, macroautophagy is the representative type of autophagy, and so it is simply called autophagy. When a cell receives an autophagy induction signal, the process occurs through several steps **(Figure 2)**. First, nucleation of the phagophore is built up from organelle membranes by the activation of the ULK1 complex combined with ULK1, ULK2, ATG13, FIP200, and ATG101 (Levy et al. 2017). The ULK1 complex then activates a

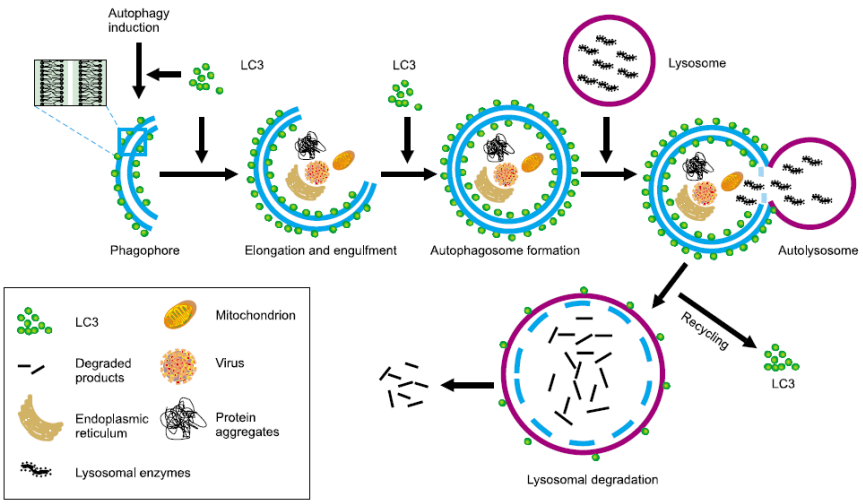

Figure 2. The process of autophagy (Jing et al. 2012).

LC3, microtubule-associated protein light chain 3.

class 3 PI3K complex combined with VPS34, ATG14, UVRAG, and AMBRA1.

Second, phagophore is expanded by combination with the ATG5–ATG12 complex and ATG16. At this stage, LC3 is recruited to phagophore. Third, autophagosome is formed when ATG4B conjugates phosphatidylethanolamine (PE) to LC3-1 and transforms LC3-1 into LC3-2 (Levy et al. 2017). Finally, autophagosome is fused with lysosome and substrates, such as dysfunctional organelles, unfolded proteins, and p62, are degraded.

Basal autophagy maintains cellular homeostasis and performs a housekeeping function; however, excessive activation of autophagy can also lead to cell death by destroying major proportions of the cytoplasm. Therefore, autophagic cell death represents an alternative approach to inhibiting the growth of apoptosis-resistant cancer cells (Jia et al. 2015). Therefore, it is important to understand the function of autophagy in specific context. Autophagic cell death differs from apoptosis in several ways, one of which is morphological character. Cells undergoing autophagic cell death do not show nuclear fragmentation and condensation, plasma membrane blebbing. From this point of view, autophagic cell death is called “type 2 programmed cell death.”

One of the most famous tumor suppressors is p53; however, it is defective in various human cancers. Because p53 is involved in many cell signaling systems, the mutation of p53 is lethal and is resistant against many drugs. Cell proliferation is regulated by p53 and leads to cell cycle arrest by the upregulation of p21, which is a cyclin dependent kinase inhibitor, while mutant p53 cannot regulate cell growth. Wild type p53 protects DNA from mutation,

which is why people describe p53 as “the guardian of the genome.”

Additionally, wild type p53 mediates apoptosis by regulating the expression of proapoptotic factors, including *Bax*, *Noxa*, and *Puma*, that could disrupt the function of anti-apoptotic factors by binding them. Moreover, p53 is involved in autophagy regulation by transcription dependent and independent mechanisms. Autophagy induction and inhibition is regulated by p53 by the transactivating of the *Dram1* and *Tigar* genes. Generally active form of p53 located in nuclear increases the expression of *Dram1*, which is a p53-dependent autophagy inducer, but cytoplasmic p53 increases the expression of *Tigar*, which is a p53-dependent autophagy inhibitor. However, the mechanisms of p53-dependent induction of autophagy are still incompletely understood.

Apigenin (4′,5,7-trihydroxyflavone) and luteolin (3′,4′,5,7-tetrahydroxy flavone) are natural flavonoids that are found in various fruits and vegetables, such as parsley, celery, chamomile, and perilla leaf (Shukla et al. 2010). The anticancer effects of apigenin and luteolin on various cancers have been determined in both in vitro and in vivo studies (Leonardi et al. 2010, Salmani et al. 2017, Wang et al. 2017). In colon cancer, the anticancer effects of apigenin and luteolin are the inhibition of proliferation by the upregulation of the *p21* gene, which induces apoptosis by the stabilization of p53, and the inhibition of invasion by the regulation of Wnt/beta-catenin signaling (Wang et al. 2000, Zhong et al. 2010, Xu et al. 2016) (Pandurangan et al. 2013). Also, phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) and mitogen-activated protein kinase (MAPK) pathways have been shown to be involved in the anticancer effects of flavonoids (Seelinger et al. 2008). Besides this,

interactions with compounds and anticancer drugs, such as ABT-263 and cisplatin, have been examined (Shi et al. 2007, Shao et al. 2013). Although apigenin induces autophagy, the mechanisms are unclear in colon cancer (Lee et al. 2014). Furthermore, the relationship of luteolin and autophagy have not been investigated in colon cancer.

Apigenin and luteolin differ in the hydroxyl group in the 3'-position of the B ring (Funakoshi-Tago et al. 2011). Apigenin has a hydroxyl group in the 3'-position of the B ring whereas luteolin does not **(Figure 3)**. These structural differences create differences in their various effects, such as anti-oxidant and anti-inflammation effects, and the efficiency of those effects (Funakoshi-Tago et al. 2011).

In colon cancer, the mechanism of apigenin-induced autophagy is poorly understood. It is also unclear whether luteolin regulates autophagy in colon cancer. Therefore, in this study, we investigated whether apigenin and luteolin could regulate autophagy in colon cancer and investigated the associated mechanism with respect to the role of p53. Our hypothesis is that apigenin and luteolin regulate autophagy through p53-dependent mechanisms.

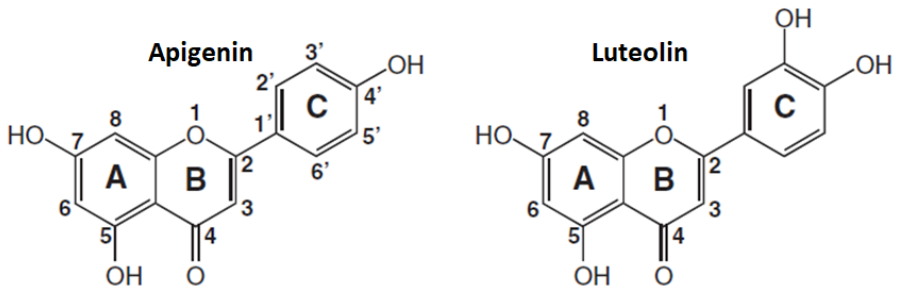

Figure 3. The structure of apigenin and luteolin (Funakoshi-Tago et al. 2011).

II. Materials and Method

1. Cell culture and treatment

Human colon cancer cells HCT-116, HT-29 and normal colon fibroblasts CCD-18Co were purchased from Korean Cell Line Bank (KCLB, Seoul, Korea). All cells were cultured in phenol-red containing Dulbecco's modified Eagle's medium (DMEM; WelGene, Korea) supplemented with 10% heat-inactivated fetal bovine serum (FBS; WelGene) and 1% streptomycin-penicillin (WelGene). The cells were maintained in a humidified incubator set to 37℃ with 5% CO2. All Cells were passaged at 70%-80% confluence using trypsin-EDTA solution.

HCT-116 and HT-29 cells seeded 0.6×10^{6}cells/well in 6-well plates for extraction of protein and RNA , 0.2×10^{6}cells /well in 12-well plates for assessment of cell viability. CCD-18Co cells were seeded 0.4× 10^5cells/well in 12-well plates for assessment of cell viability. After 24h from cell seeding, the culture medium was exchanged to serum free DMEM and maintained for 16h. And then, cells were treated with Luteolin (0-40 μmol/L dissolved in DMSO) or Apigenin (0-25 μmol/L dissolved in DMSO) for 24h. Chloroquine, were dissolved in DMSO. Treatment with DMSO for dissolving reagent served as a control.

2. Assessment of cell viability by trypan blue exclusion assays

Cell viability was assessed by trypan blue exclusion assays. After treatment with Luteolin (Sigma, USA), Apigenin (Sigma, USA) for 24h, the cells were washed with PBS for twice and trypsinized. Cells were then stained with 0.4% trypan blue dye (Sigma, USA) and counted using hemocytometer. The effect of each treatment on cell viability was assessed by the relative value compared with control value.

3. Total RNA extraction and quantitative real-time PCR analysis

Treated cells were washed twice with PBS and added RNA isoplus (Takara, Japan) and then scraped. for RNA extraction. The quality of RNA was confirmed using spectrophotometer at 260/280nm and electrophoresis. cDNA was synthesized using 2μg of total RNA with Superscript II Reverse Transcriptase (Invitrogen, USA). SYBR Green PCR Master Mix (Applied Biosystems) mixed with cDNA according to supplier's protocol and measured relative mRNA expression level using Step One Real time PCR system. *Acbt* was used as a reference gene and relative gene expression levels was quantified according to 2^(-ΔΔCt) method. To determine mRNA levels of spliced *Xbp1 (Xbp1s*), semi-quantitative PCR was used as previously described (Kim et al. 2011). The primer sequences are described in Supplementary Table 1. the sequences of used primer in experiments were presented at **Table 1**.

Table 1. The sequences of primer for real time PCR

Gene		Sequence (5′→3′)
Quantitative PCR		
Chop	forward	ATGGCAGCTGAGTCATTGCCTTTC
	reverse	AGAAGCAGGGTCAAGAGTGGTGAA
Noxa	forward	GCTGGAAGTCGAGTGTGCA
	reverse	CCTGAGCAGAAGAGTTTGGA
p21	forward	GACAGCAGAGGAGAGCCAT
	reverse	TGGAGTGGTAGAAATCTGTCAT
Acbt	forward	TGCCAATCTCATCTTGTTTTCT
	reverse	GTCCACCTTCCAGCAGATGT

4. Semi-quantitative RT-PCR

To examine expression of spliced *Xbp-1* form, ER stress indicator, we amplified cDNA by *Xbp-1* primer. And then, treated restrict enzyme PST1 into cDNA products to check spliced form clearly from unspliced form. Amplified products were separated through an agarose gel and visualized with loading star staining. The sequences of used primer were presented at **Table 2**.

Table 2. The sequences of primer for semi-quantitative PCR

Semi-quantitative PCR		
Xbp1	forward	AAACAGAGTAGCAGCTCAGACTGC
(68℃, 38 cycle)	reverse	TCCTTCTGGGTAGACCTCTGGGAG

5. Total protein extraction and western blotting analysis

After treatment, the cells were washed twice with PBS and scraped. Total protein was extracted by using ice-cold lysis buffer containing 50mM Hepes-KOH (pH 7.5), 150mM NaCl, 1mM EDTA (pH 8.0), 1mM EDTA (pH 8.0), 1mM NaF, 10mM β-glycerophosphate, 0.1mM Na3Vo4, 0.1% Tween 20, 10% glycerol, 1mM DTT, and the protease inhibitor cocktail(Sigma). Protein concentration of lysates was determined with Protein Assay Dye Reagent (Bio-Rad, USA). Equal amounts of protein were loaded into lanes of a SDS PAGE gel, electrophoresed, and blotted onto a PVDF membrane (Millipore, USA). After blocking with 5% nonfat milk or bovine serum albumin they were probed with a specific primary antibody as follows: anti-Beclin1 (Santa Cruz Biotechnology, USA), anti-microtubule-associated protein light chain 3 (LC3; Novus Biology, USA), anti-poly (ADP-ribose) polymerase (PARP; Cell Signaling, USA), anti-proliferation cell nuclear antigen (PCNA; Santa Cruz Biotechnology), anti-p53 (Santa Cruz Biotechnology), anti-p-p53 (Cell Signaling), anti-p62/SQSTM1 (Santa Cruz Biotechnology), or anti-heat shock protein 70 (HSC70; Cell Signaling Membranes were then incubated with a horseradish peroxidase-linked secondary antibody for chemiluminescent detection. and these bands visualized by X-ray film and then the bands intensity was quantified by using Quantity One software (Bio Rad, USA).

6. Measurement of autophagic flux

Autophagic flux was detected by preventing lysosomal degradation using chloroquine (CQ; Sigma), which inhibits pH-dependent lysosomal protease activity and leads to the further accumulation of LC3-II (Fass, Shvets et al. 2006). In the absence or presence of 10μmol/L. CQ, cells were overloaded with apigenin and luteolin for 24 h as described above. Autophagic flux was determined by subtracting the densitometry value of normalized LC3-II in the sample in the absence of CQ from the densitometry value of normalized LC3-II in the sample in the presence of CQ.

7. Statistical analysis

Statistical analysis was conducted using SPSS software (version 22.0). One-way or two-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Student's t-test was used for two-group comparison. All data were presented as mean ± SEM, and differences were considered statistically significance at $P<0.05$.

III. Results

1. Apigenin and luteolin reduced the viability of colon cancer cells HCT-116 and HT-29 but not normal colon cells CCD-18CO

To examine the concentrations of apigenin and luteolin that did not affect the viability of normal colon cells, human colon cancer cells HCT-116 and HT-29 and human colon fibroblast cells CCD-18Co were treated with various concentrations for 24 h. The range of the concentrations was 0–25 μmol/L for apigenin and 0–20 μmol/L for luteolin. Additionally, we assessed the effect on the viability of the cells at a range of concentrations. As a result, the viability of HCT-116 p53 wild type colon cancer cell, decreased in a dose dependent manner. However, the viability of HT-29 p53 mutant colon cancer cells, appeared resistant to the effects of luteolin and high concentrations of apigenin **(Figure 4)**. Indeed, many p53 mutant cancer cells appear resistant to drugs and this is accompanied by poor prognosis (Oren et al. 2010). Remarkably, at low concentrations of apigenin (1 μmol/L and 5 μmol/L), HT-29 decreased in a dose dependent manner. These results suggests the possibility that luteolin and high concentrations of apigenin induce p53-dependent cell death, but p53-independent cell death was caused at low concentrations of apigenin in colon cancer cells.

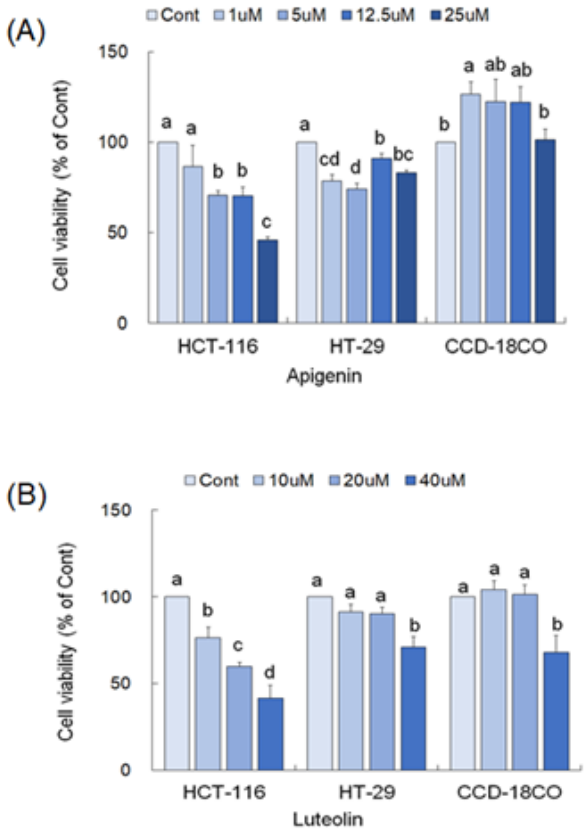

Figure 4. The cytotoxicity effect of apigenin and luteolin on cancerous cells HCT-116 and HT-29 and non-cancerous cells CCD-18Co.

(A) Colon cancer cells and fibroblast cells were treated with apigenin (0–25 μmol/L, DMSO 0.2%). (B) Colon cancer cells and fibroblast cells were treated with luteolin (0–40 μmol/L, DMSO 0.2%). The cytotoxicity of apigenin and luteolin was assessed by trypan blue exclusion assay. Data were presented as mean ± standard error of mean SEM (n = 3–9). Bars with different superscripts are significantly different (one-way analysis of variance ANOVA followed by Duncan multiple range test, $P < 0.05$).

2. Apigenin and luteolin activated p53 by phosphorylation

To investigate the involvement of p53 in the anticancer mechanisms of apigenin and luteolin, we examined the expression of phosphorylated p53 proteins in the presence of apigenin and luteolin. We could detect the phosphorylation of p53 at Ser15 in HCT-116 cells treated with apigenin 25 μmol/L **(Figure 5A)** and luteolin 20 μmol/L **(Figure 5C)**. But HT-29 treated with apigenin and luteolin did not changed **(Figure 5B, D)**. Although the total p53 did not increase in HCT-116 cells in the presence of apigenin 25 μmol/L, luteolin 20 μmol/L did increase the total p53 in HCT-116. An increase in the phosphorylation of p53 leads to the stability of p53 from MDM2 (Zheleva et al. 2003).

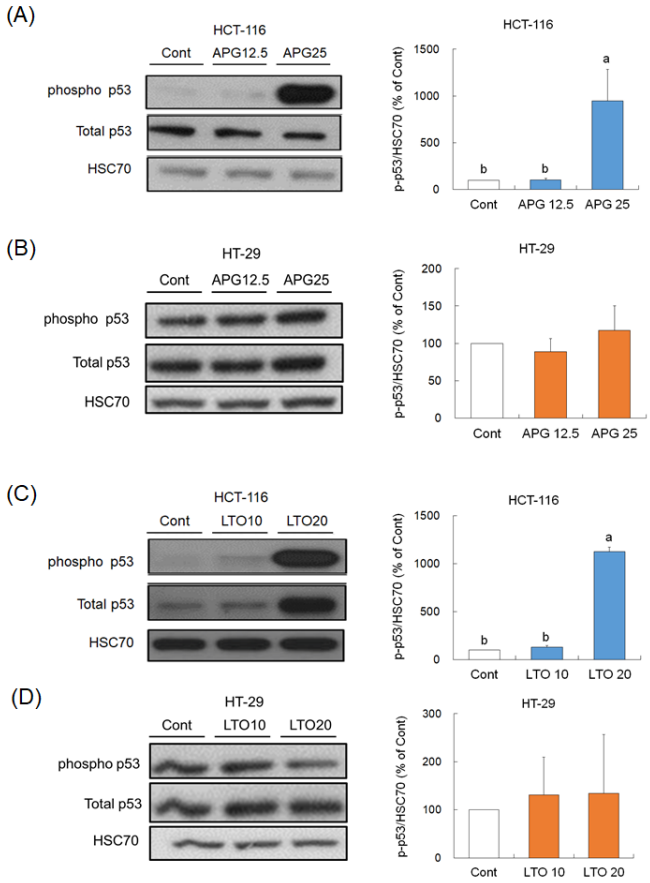

Figure 5. The expression of phosphorylated p53 protein in HCT-116 and HT-29 treated with luteolin and high concentration of apigenin.

(A, B) The expression of phosphorylated p53 was determined by immunoblotting in HCT-116 and HT-29 treated with apigenin. (C, D) The expression of phosphorylated p53 was determined by immunoblotting in HCT-116 and HT-29 treated with luteolin. HSC70 was used to normalize the data. All data were presented as mean ± SEM (n = 3). Bars with different superscripts are significantly different (one-way ANOVA followed by Duncan multiple range test, $P < 0.05$).

3. Luteolin and high concentrations of apigenin-induced p53-dependent apoptosis and cell cycle arrest

To investigate whether p53-dependent apoptosis and the inhibition of proliferation were induced along with the activation of p53 by luteolin and high concentrations of apigenin, we examined the expression of p53-dependent apoptosis and the inhibition of proliferation indicators in the mRNA level, such as *Noxa* and *p21* in HCT-116, and HT-29 **(Figure 6)**.

Indeed, the protein expression of cleaved Parp, which is a well-recognized marker for apoptosis, increased **(Figure 7)**, and proliferating cell nuclear antigen (PCNA), which is a marker for proliferation, decreased in HCT-116 treated with apigenin **(Figure 8A)** and luteolin **(Figure 8C)**, but the expression of HT-29 was not **(Figure 8B, D)**. These results clearly indicate p53 involvement in anticancer mechanisms, including apoptosis and the proliferation inhibition of luteolin and high concentrations of apigenin.

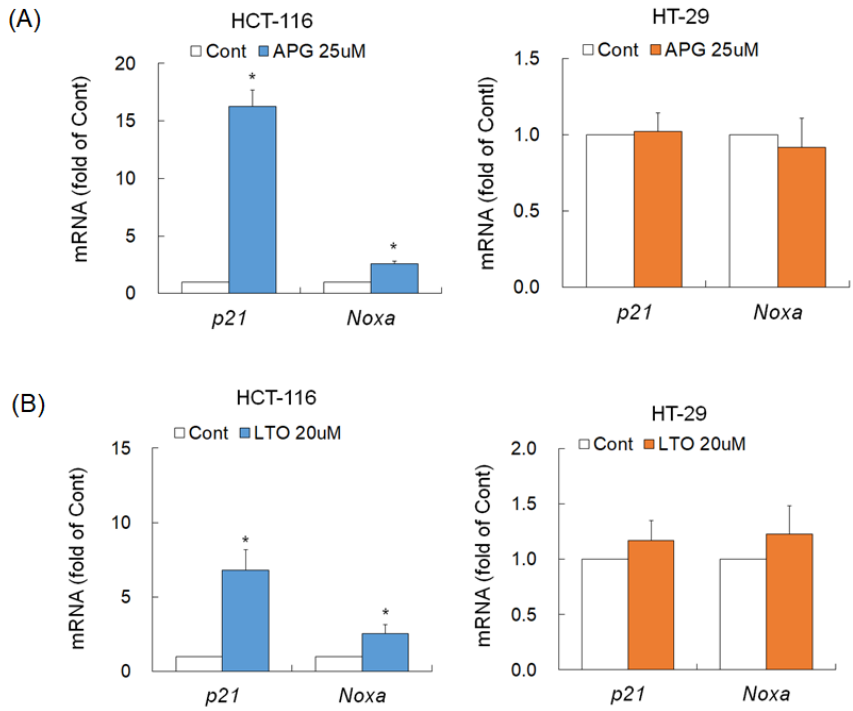

Figure 6. The expression of p53 activation indicator.

(A) The mRNA expression of *Noxa* and *p21* in HCT-116 and HT-29 treated with apigenin. (B) The mRNA expression of *Noxa* and *p21* in HCT-116 and HT-29 treated with luteolin. HSC70 was used to normalize the data. Data were presented as mean ± SEM (n = 3–5). Bars with different superscripts are significantly different (one-way ANOVA followed by Duncan multiple range test, $P < 0.05$).

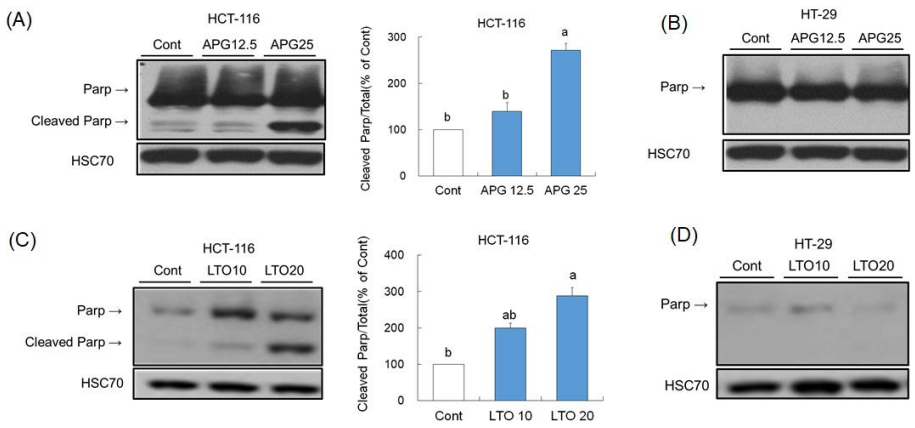

Figure 7. The expression of apoptosis indicator in HCT-116 and HT-29 treated with luteolin and high concentrations of apigenin.

(A, B) The protein expression of cleaved Parp in HCT-116 and HT-29 treated with apigenin. (C, D) The protein expression of cleaved Parp in HCT-116 and HT-29 treated with luteolin. HSC70 was used to normalize the data. Data were presented as mean ± SEM (n = 3). Bars with different superscripts are significantly different (one-way ANOVA followed by Duncan multiple range test, $P < 0.05$).

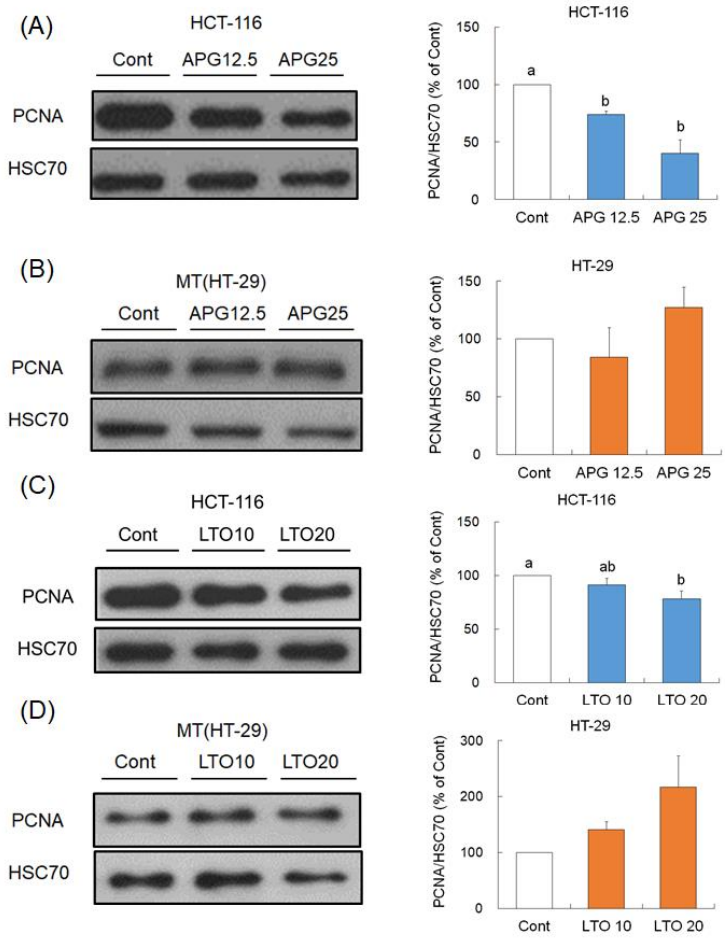

Figure 8. The expression of cell cycle arrest indicator in HCT-116 and HT-29 treated with luteolin and high concentrations of apigenin.

(A, B) The protein expression of PCNA in HCT-116 and HT-29 treated with apigenin. (C, D) The protein expression of PCNA in HCT-116 and HT-29 treated with luteolin. HSC70 was used to normalize the data. Data were presented as mean ± SEM (n = 3). Bars with different superscripts are significantly different (one-way ANOVA followed by Duncan multiple range test, $P < 0.05$).

4. Low concentrations of apigenin-induced p53-independent cell cycle arrest

Additionally, to confirm the reason for the decrease in HT-29 treated with low concentrations of apigenin, we determined the expression of PCNA for the examination of inhibition of proliferation.

As a result, when HCT-116 and HT-29 were treated with low concentrations of apigenin, the expression of PCNA decreased in HT-29 **(Figure 9B)**. In other words, p53-independent proliferation inhibition was induced in a different manner than the high concentration circumstance.

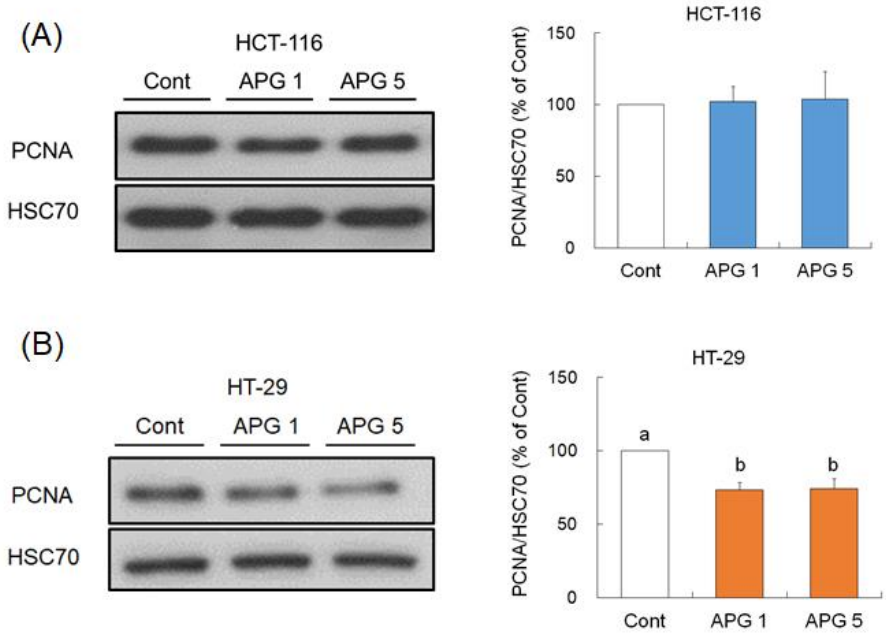

Figure 9. The expression of apoptosis and cell cycle arrest indicator in HCT-116 and HT-29 treated with low concentrations of apigenin.

(A) The protein expression of PCNA in HCT-116 treated with low concentrations of apigenin. (B) The protein expression of PCNA in HT-29 treated with low concentrations of apigenin. HSC70 was used to normalize the data. Data were presented as mean ± SEM (n = 3). Bars with different superscripts are significantly different (one-way ANOVA followed by Duncan multiple range test, $P < 0.05$).

5. Apigenin and luteolin increased ER stress in a p53-dependent manner

One of the mechanisms that can affect p53-dependent apoptosis is ER stress. To determine whether ER stress-induced apoptosis was caused, the mRNA expression of *Chop* indicator was examined by real time PCR when treated with apigenin and luteolin. As a result, the mRNA expression of *Chop* tends to increase in HCT-116 treated with apigenin and luteolin but not in HT-29 **(Figure 10 A, B)**. In addition, spliced *Xbp-1* as an ER stress indicator was examined by semi-quantitative PCR in HCT-116 treated with apigenin and luteolin. The expression of HCT-116 samples treated with apigenin and luteolin was higher than the control samples, but HT-29 samples were not changed **(Figure 10 C)**. Consequently, the mRNA levels of *Chop,* a gene encoding C/EBP homologous protein involved in ER stress-mediated apoptosis, and *Xbp-1* tended to increase. No significant changes were observed in HT-29 cells in response to luteolin and high concentrations of apigenin. These results showed that luteolin and high concentrations of apigenin induce ER stress, and apigenin leads to ER stress-induced apoptosis.

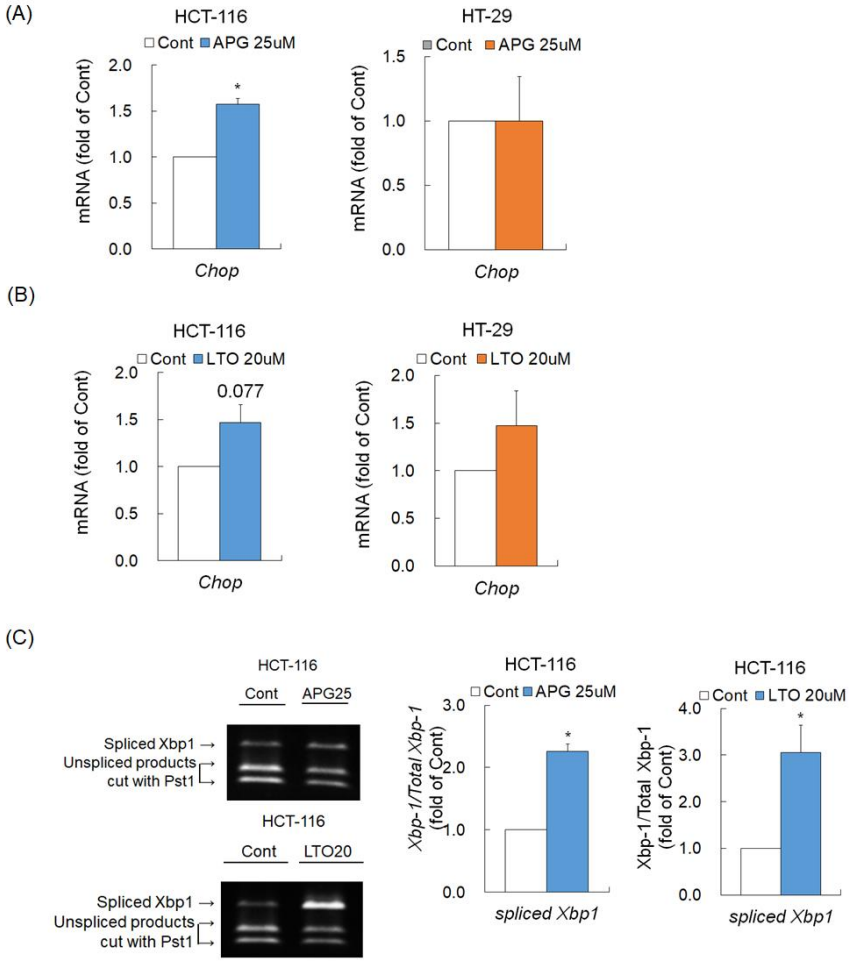

Figure 10. Apigenin- and luteolin-induced ER stress in p53 wild type colon cancer cells.

(A) The mRNA expression of *Chop* in colon cancer cells treated with apigenin. (B) The mRNA expression of *Chop* in colon cancer cells treated with luteolin. (C) The mRNA expression of *Xbp-1* in colon cancer cells treated with apigenin and luteolin. Quantification of the expression of spliced *Xbp-1* was calculated by spliced *Xbp-1*/Total *Xbp-1*. Data were presented as mean ± SEM (n = 3–4). **P* < 0.05 compared with control (Student′s *t*-test).

6. Apigenin- and luteolin-induced p53-dependent autophagy

To determine whether luteolin and high concentrations of apigenin affect autophagy in colon cancer cells, we measured the protein levels of several autophagy markers. Luteolin treatment at 10 and 20 μmol/L and apigenin at 12.5 and 25 μmol/L significantly increased the expression of LC3-II in HCT-116 **(Figure 11A, C)** but did not changed in HT-29 **(Figure 11B, D)**. During autophagy, pro-LC3 is proteolytically cleaved to LC3-I, a cytosolic form with an exposed glycine at the carboxy terminal. LC3-I covalently links to the highly lipophilic phosphatidylethanolamine to produce LC3-II, which is incorporated into autophagosome membranes (Barth, Glick et al. 2010). p62 also increased when HCT-116 and HT-29 were treated with luteolin **(Figure 12C, D)** but did not change in HCT-116 and HT-29 treated with high concentrations of apigenin **(Figure 12A, B).**

Increased LC3-II levels might indicate an increase in autophagosome synthesis but could also be due to a defective clearance of autophagosomes (Barth, Glick et al. 2010). The difference in LC3-II protein levels in the presence or absence of CQ was not significantly different between the control and luteolin- or apigenin-treated cells, which suggests that autophagic flux was not impaired by luteolin and apigenin **(Figure 13)**.

To confirm the function of autophagy induced by apigenin and luteolin, CQ, an inhibitor of autophagome-lysosome fusion stage, was treated, and examined changes in cell viability and apoptosis. Despite autophagy inhibition, the addition of CQ *per se* did not induce cell death as much as luteolin or apigenin. Furthermore, the inhibition of autophagosome clearance did not alter apoptosis **(Figure 14A, B)** and cytotoxicity **(Figure 14C, D)** in HCT-116 cells.

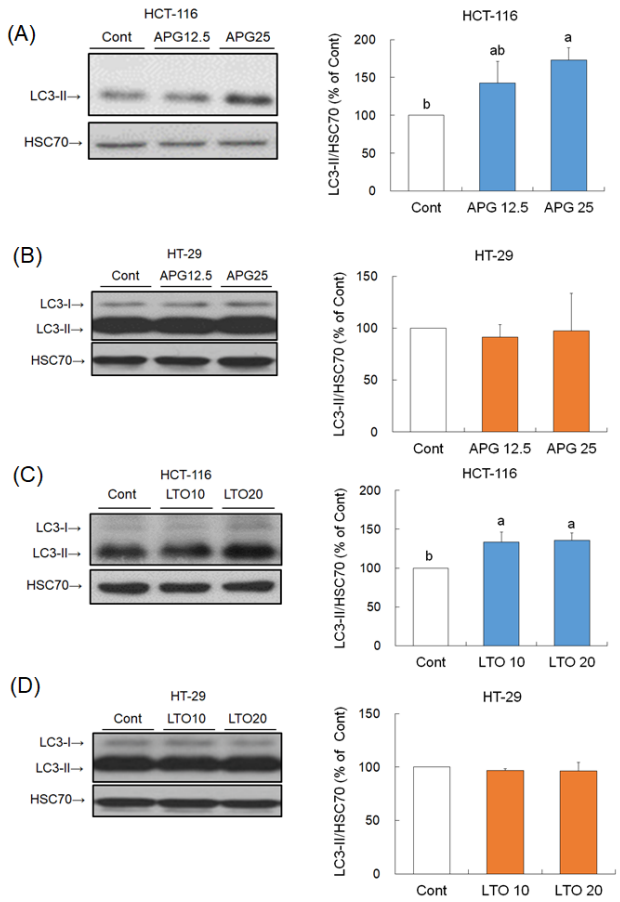

Figure 11. Apigenin and luteolin increased the protein expression of LC3-II, which is a marker of autophagy induction in colon cancer cells in a p53-dependent manner.

(A, B) The protein levels of LC3-II of HCT-116 and HT-29 treated with apigenin were determined by immunoblotting. Data were presented as mean ± SEM (n = 4). (C, D) The protein levels of LC3-II of HCT-116 and HT-29 treated with luteolin were determined by immunoblotting. Data were presented as mean ± SEM (n = 3). Bars with different superscripts are significantly different (one-way ANOVA followed by Duncan multiple range test, $P < 0.05$).

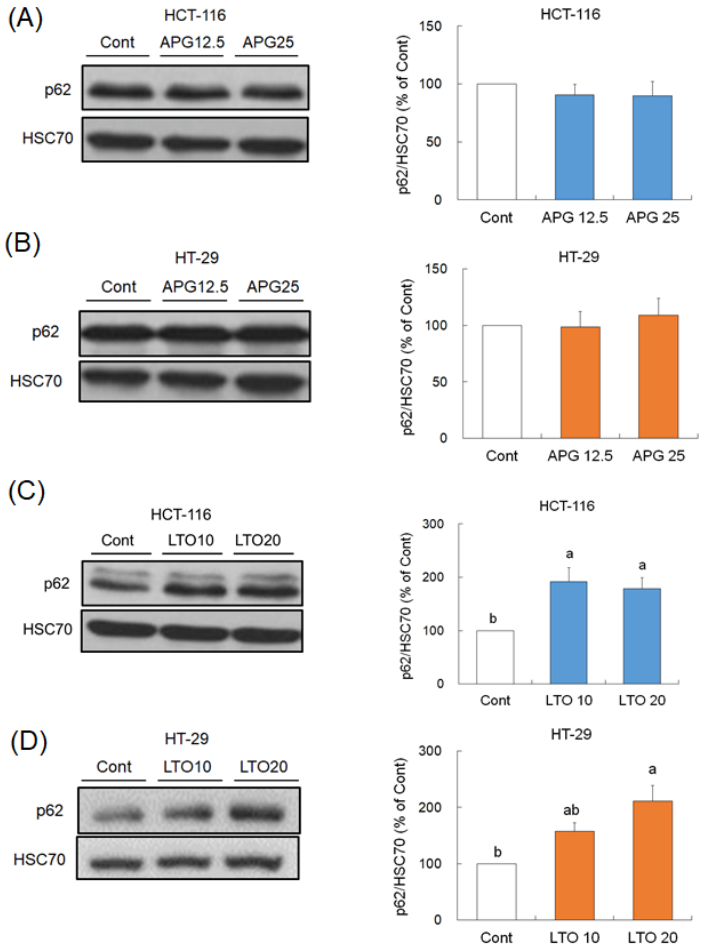

Figure 12. Apigenin and luteolin increased the protein expression of p62, which is a marker for autophagosome–lysosome fusion in colon cancer cells. (A, B) The protein levels of p62 of HCT-116 and HT-29 treated with apigenin were determined by immunoblotting. Data were presented as mean ± SEM (n = 3). (C, D) The protein levels of p62 of HCT-116 and HT-29 treated with luteolin were determined by immunoblotting. Data were presented as mean ± SEM (n = 3). Bars with different superscripts are significantly different (one-way ANOVA followed by Duncan multiple range test, $P < 0.05$).

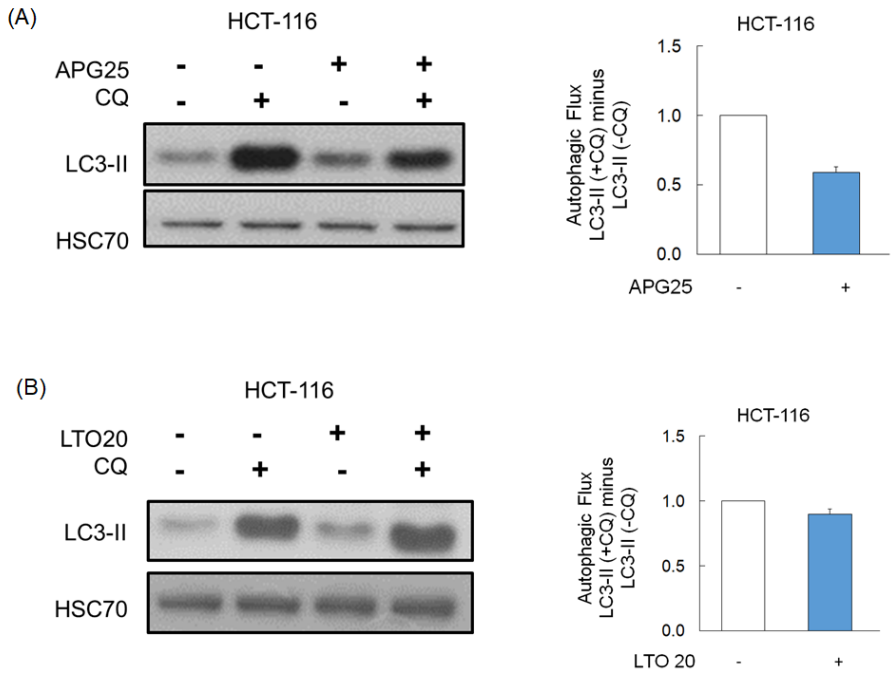

Figure 13. An increase in LC3-II was not caused by the inhibition of fusion with autophagosome and lysosome. (A) Apigenin-regulated autophagic flux was determined by subtracting the densitometry value of normalized LC3-II in the sample in the absence of CQ from the densitometry value of normalized LC3-II in the sample in the presence of CQ. (B) Luteolin-regulated autophagic flux was determined by subtracting the densitometry value of normalized LC3-II in the sample in the absence of CQ from the densitometry value of normalized LC3-II in the sample in the presence of CQ. All data were presented as mean ± SEM (n = 3).

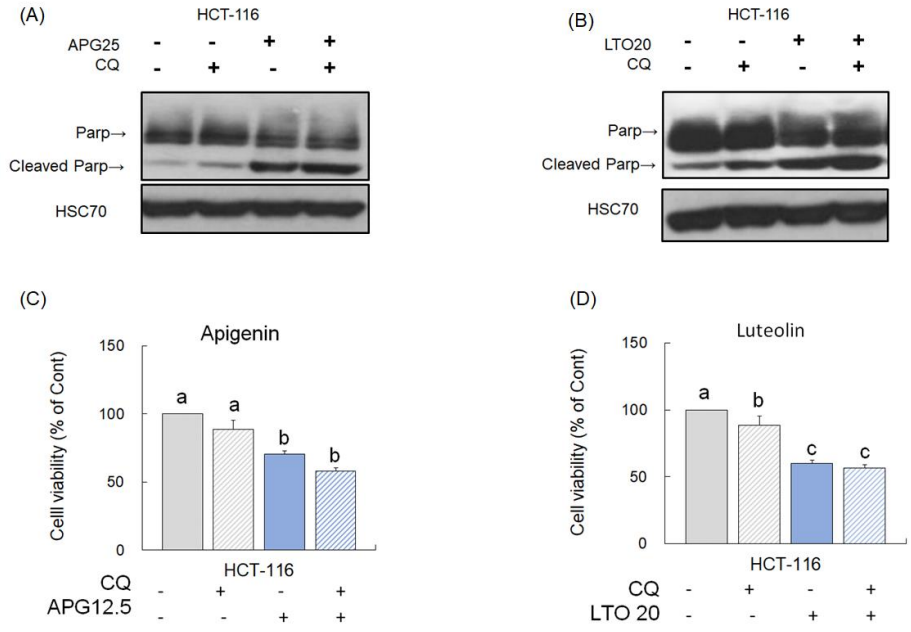

Figure 14. Autophagy inhibition by chloroquine did not affect apigenin- and luteolin-induced apoptosis and viability reduction in colon cancer cells. (A) Apigenin-induced apoptosis in the presence or absence of CQ was determined by protein level. (B) Luteolin-induced apoptosis in the presence or absence of CQ was determined by protein level. (C) Apigenin-induced reduction of viability in the presence or absence of CQ was determined by trypan blue exclusion assay. (D) Luteolin-induced reduction of viability in the presence or absence of CQ was determined by trypan blue exclusion assay. HSC70 was used to normalize the data. All data were presented as mean ± SEM (n = 3) Bars with different superscripts are significantly different (one-way ANOVA followed by Duncan multiple range test, $P < 0.05$).

IV. Discussion

Apigenin and luteolin have been shown to have anticancer effects in various types of cancer (Selvendiran et al. 2006). The regulation of autophagy, in addition to apoptosis, is considered as an anticancer mechanism because autophagy conducts a pivotal function in survival. Several studies suggest that apigenin and luteolin contribute to the inhibition of proliferation and invasion and the induction of apoptosis in colon cancer (Seelinger et al. 2008). In addition, one study suggests that apigenin-induced autophagy in colon cancer (Lee et al. 2014). However, the mechanism of autophagy involved in apigenin and the relationship between luteolin and autophagy in colon cancer cells are unclear. In this study, we tried to clarify the mechanism of autophagy induced by apigenin and find whether luteolin that is structurally similar to apigenin could regulate autophagy in colon cancer cells. Because several transcriptional targets of p53 can promote autophagy, we examined the regulation of autophagy using two different colon cancer cells ; one with wild type p53 and the other with mutant p53.

First, we examined the effect of apigenin and luteolin on the viability of colon cancer cells and normal colon cells to identify the treatment concentration that does not affect the viability of normal colon cancer cells. We found that 0–25 μmol/L of apigenin and 0–20 μmol/L of luteolin do not affect the viability of normal colon cells. This difference in sensitivity to anticancer agents between cancer cells and normal cells results from the difference in response against stimulation (Fisher 1994). When stimulation is induced, cell cycle arrest occurs and the repair system is operated in normal

cells. In contrast, apoptosis is operated and leads to cell death in cancer cells. This is why normal colon cells with wild type p53 were not affected by apigenin and luteolin.

In addition, we should examine whether the range of treatment concentrations is practical. In a previous study, the intake of total flavonoids, including quercetin, kaempferol, myricetin, luteolin, and apigenin, in Europe was approximately 400 mg/day. Among these, the average intake of luteolin was approximately 1 mg/day (Vogiatzoglou et al. 2015). The luteolin intake of Chinese adults was 1.06–3.85 mg/day (Thilakarathna et al. 2013). When 50 μg apigenin, which has a similar structure to luteolin, was fed by oral gavage for 8 weeks, plasma concentrations were 1 μmol/L (Shukla et al. 2006). After a single oral administration of 10 mg apigenin to male mice radiolabeled apigenin was recovered at approximately 1.3% in the blood and approximately 9.2 % in the intestine (Gradolatto et al. 2005). In other words, when mice intake 100 μg of apigenin, apigenin exist at a concentration of approximately 20 μmol/L in the intestine. These studies indicate that the concentration we used is practical.

The viability of the p53 wild type cell line decreased in a dose dependent manner, while the viability of HT-29, which was the p53 mutant cell line, showed resistance at high concentrations of apigenin (12.5 and 25 μmol/L), as well as apigenin and luteolin decreased viability of cancerous cells specifically. This difference results from the mutation of p53. Phosphorylation at a specific site, including Ser15, activates p53 (Ashcroft et al. 1999). Because of the phosphorylation of p53, the ubiquitin E3 ligase cannot bind to a phosphorylated form of p53, which results in increased stability and transactivation activity

(Fridman et al. 2003). As a result, the stability of p53 is increased, and their functions are activated—for example, the transactivation of proapoptotic factors and growth inhibition factors, such as *Noxa*, *Bax*, and *p21*. In contrast, HT-29 is mutated in codon 273 of the p53 gene, which results in an Arg to His substitution. Because the mutated site is involved in the DNA binding function, HT-29 loses its transactivation function (Tan et al. 2015). Also, the mutation of p53 gains additional functions, such as interaction with transcription factors related to anti-apoptosis and proliferation. For these reasons, many p53 mutant cancer cells appear resistant to drugs, and this results in poor prognosis (Oren et al. 2010).

Apigenin 25 μmol/L and luteolin 20 μmol/L increased the expression of phosphorylated p53, but the expression of total p53 also increased in HCT-116 treated with luteolin. In a previous study, apigenin could increase total p53 when HCT-116 was treated with 50 μmol/L (Lee et al. 2014). This indicates that there is a difference in p53 activation efficiency between apigenin and luteolin caused by structural differences.

When p53 is activated, various factors related with apoptosis and proliferation, such as *Noxa* and *p21*, are transactivated (Wawryk-Gawda et al. 2014). In this study, according to the activation of p53, the mRNA expression levels of *Noxa* and *p21* increased significantly in HCT-116 but did not change in HT-29 treated with luteolin and high concentrations of apigenin.

ER stress could also interact with p53. A previous study explained that ER stress could be affected by p53 (Byun et al. 2015). To confirm the occurrence of ER stress, we examined the expression of spliced *Xbp-1* and *Chop* in colon cancer cells treated with apigenin and luteolin. It was found that spliced *Xbp-1*

was increased in HCT-116 but not HT-29 by apigenin and luteolin. Additionally, apigenin increased the expression of *Chop*, which is indicator of apoptosis induced by ER stress. This result means that ER stress is also caused by treatment with apigenin and luteolin in a p53-dependent manner.

Apigenin showed an unusual anticancer mechanism that does not depend on treatment concentrations. In a previous study, the fact that apigenin 10 μmol/L induces p53-independent cell cycle arrest was investigated. This study also confirmed that low concentrations of apigenin-induced p53-independent cell cycle arrest. The expression level of PCNA inhibition of proliferation decreased in HT-29 treated with low concentrations of apigenin. These results support the hypothesis that low concentrations of apigenin induce p53-independent inhibition proliferation. Additionally, we compared p53 dependency on treatment concentrations, and we found that high concentrations of apigenin-induced p53-dependent apoptosis and cell cycle arrest.

Oncogenic stress stabilizes and activates p53 resulting in autophagy stimulation via both transcription-independent pathways, such as AMP-activated protein kinase (AMPK) activation and mammalian target of rapamycin (mTOR) inhibition, and transcription-dependent pathways by upregulation of *Pten*, *Tsc1*, and *Dram* genes. In contrast, the genetic or chemical inhibition of p53 or the proteasomal depletion of p53 during starvation and ER stress are shown to activate autophagy through transcription-independent mechanisms (Levine et al. 2008). Therefore, further studies would be needed to investigate the role of autophagy induction in this setting.

In the present study, autophagy as well as apoptosis were induced in response to apigenin and luteolin in a p53-dependent manner. Recently, it has been

shown that there is interaction between anti-apoptotic protein Bcl-2 and the autophagy protein, Beclin-1. Beclin1 may play an important role by mediating the localization of other autophagy proteins to the preautophagosomal membrane and participates in autophagosome formation after dissociation with Bcl-2 (Kouroku et al. 2007).

In the present study, we observed increases in both LC3-II and in response to apigenin and luteolin, which suggests the induction of autophagosome formation.

However, increased LC3-II levels might indicate an increase in autophagosome synthesis, although this could also be due to a defective clearance of autophagosomes (Barth et al. 2010). LC3-II protein levels in the presence or absence of CQ were not significantly different between control and luteolin-treated cells, which suggests that autophagic flux was not impaired by apigenin and luteolin.

In spite of autophagy inhibition, the addition of CQ per se did not induce cell death as much as luteolin and apigenin. Furthermore, the inhibition of autophagosome clearance did not alter apoptosis and cytotoxicity in HCT-116 cells.

In conclusion, the present data showed that luteolin and apigenin could inhibit the growth of colon cancer cells through p53-dependent apoptosis and autophagy.

Reference

Ashcroft, M., M. H. Kubbutat and K. H. Vousden (1999). "Regulation of p53 function and stability by phosphorylation." Mol Cell Biol **19**(3): 1751-1758.

Barth, S., D. Glick and K. F. Macleod (2010). "Autophagy: assays and artifacts." J Pathol **221**(2): 117-124.

Byun, S., T. Namba and S. W. Lee (2015). "Losing p53 loosens up ER-stress." Aging (Albany NY) **7**(11): 895-896.

Fisher, D. E. (1994). "Apoptosis in cancer therapy: crossing the threshold." Cell **78**(4): 539-542.

Fridman, J. S. and S. W. Lowe (2003). "Control of apoptosis by p53." Oncogene **22**(56): 9030-9040.

Funakoshi-Tago, M., K. Nakamura, K. Tago, T. Mashino and T. Kasahara (2011). "Anti-inflammatory activity of structurally related flavonoids, Apigenin, Luteolin and Fisetin." Int Immunopharmacol **11**(9): 1150-1159.

Gradolatto, A., J. P. Basly, R. Berges, C. Teyssier, M. C. Chagnon, M. H. Siess and M. C. Canivenc-Lavier (2005). "Pharmacokinetics and metabolism of apigenin in female and male rats after a single oral administration." Drug Metab Dispos **33**(1): 49-54.

Ichim, G. and S. W. Tait (2016). "A fate worse than death: apoptosis as an oncogenic process." Nat Rev Cancer **16**(8): 539-548.

Jia, Z., P. Nallasamy, D. Liu, H. Shah, J. Z. Li, R. Chitrakar, H. Si, J. McCormick, H. Zhu, W. Zhen and Y. Li (2015). "Luteolin protects against vascular inflammation in mice and TNF-alpha-induced monocyte adhesion to

endothelial cells via suppressing IKappaBalpha/NF-kappaB signaling pathway." J Nutr Biochem **26**(3): 293-302.

Jing, K. and K. Lim (2012). "Why is autophagy important in human diseases?" Exp Mol Med **44**(2): 69-72.

Jung, K. W., Y. J. Won, C. M. Oh, H. J. Kong, D. H. Lee, K. H. Lee and R. Community of Population-Based Regional Cancer (2017). "Cancer Statistics in Korea: Incidence, Mortality, Survival, and Prevalence in 2014." Cancer Res Treat **49**(2): 292-305.

Kim, J., Y. J. Park, Y. Jang and Y. H. Kwon (2011). "AMPK activation inhibits apoptosis and tau hyperphosphorylation mediated by palmitate in SH-SY5Y cells." Brain Res **1418**: 42-51.

Kouroku, Y., E. Fujita, I. Tanida, T. Ueno, A. Isoai, H. Kumagai, S. Ogawa, R. J. Kaufman, E. Kominami and T. Momoi (2007). "ER stress (PERK/eIF2alpha phosphorylation) mediates the polyglutamine-induced LC3 conversion, an essential step for autophagy formation." Cell Death Differ **14**(2): 230-239.

Lee, Y., B. Sung, Y. J. Kang, D. H. Kim, J. Y. Jang, S. Y. Hwang, M. Kim, H. S. Lim, J. H. Yoon, H. Y. Chung and N. D. Kim (2014). "Apigenin-induced apoptosis is enhanced by inhibition of autophagy formation in HCT116 human colon cancer cells." Int J Oncol **44**(5): 1599-1606.

Leonardi, T., J. Vanamala, S. S. Taddeo, L. A. Davidson, M. E. Murphy, B. S. Patil, N. Wang, R. J. Carroll, R. S. Chapkin, J. R. Lupton and N. D. Turner (2010). "Apigenin and naringenin suppress colon carcinogenesis through the aberrant crypt stage in azoxymethane-treated rats." Exp Biol Med (Maywood) **235**(6): 710-717.

Levine, B. and J. Abrams (2008). "p53: The Janus of autophagy?" Nat Cell

Biol **10**(6): 637-639.

Levy, J. M. M., C. G. Towers and A. Thorburn (2017). "Targeting autophagy in cancer." Nat Rev Cancer **17**(9): 528-542.

Maiuri, M. C., E. Zalckvar, A. Kimchi and G. Kroemer (2007). "Self-eating and self-killing: crosstalk between autophagy and apoptosis." Nat Rev Mol Cell Biol **8**(9): 741-752.

Marino, G., M. Niso-Santano, E. H. Baehrecke and G. Kroemer (2014). "Self-consumption: the interplay of autophagy and apoptosis." Nat Rev Mol Cell Biol **15**(2): 81-94.

Oren, M. and V. Rotter (2010). "Mutant p53 gain-of-function in cancer." Cold Spring Harb Perspect Biol **2**(2): a001107.

Pandurangan, A. K., P. Dharmalingam, S. K. Sadagopan, M. Ramar, A. Munusamy and S. Ganapasam (2013). "Luteolin induces growth arrest in colon cancer cells through involvement of Wnt/beta-catenin/GSK-3beta signaling." J Environ Pathol Toxicol Oncol **32**(2): 131-139.

Salmani, J. M. M., X. P. Zhang, J. A. Jacob and B. A. Chen (2017). "Apigenin's anticancer properties and molecular mechanisms of action: Recent advances and future prospectives." Chin J Nat Med **15**(5): 321-329.

Seelinger, G., I. Merfort, U. Wolfle and C. M. Schempp (2008). "Anti-carcinogenic effects of the flavonoid luteolin." Molecules **13**(10): 2628-2651.

Selvendiran, K., H. Koga, T. Ueno, T. Yoshida, M. Maeyama, T. Torimura, H. Yano, M. Kojiro and M. Sata (2006). "Luteolin promotes degradation in signal transducer and activator of transcription 3 in human hepatoma cells: an implication for the antitumor potential of flavonoids." Cancer Res **66**(9): 4826-4834.

Shao, H., K. Jing, E. Mahmoud, H. Huang, X. Fang and C. Yu (2013). "Apigenin sensitizes colon cancer cells to antitumor activity of ABT-263." Mol Cancer Ther **12**(12): 2640-2650.

Shi, R., Q. Huang, X. Zhu, Y. B. Ong, B. Zhao, J. Lu, C. N. Ong and H. M. Shen (2007). "Luteolin sensitizes the anticancer effect of cisplatin via c-Jun NH2-terminal kinase-mediated p53 phosphorylation and stabilization." Mol Cancer Ther **6**(4): 1338-1347.

Shukla, S. and S. Gupta (2010). "Apigenin: a promising molecule for cancer prevention." Pharm Res **27**(6): 962-978.

Shukla, S. and S. Gupta (2006). "Molecular targets for apigenin-induced cell cycle arrest and apoptosis in prostate cancer cell xenograft." Mol Cancer Ther **5**(4): 843-852.

Siegel, R. L., K. D. Miller and A. Jemal (2018). "Cancer statistics, 2018." CA Cancer J Clin **68**(1): 7-30.

Tan, B. S., K. H. Tiong, H. L. Choo, F. F. Chung, L. W. Hii, S. H. Tan, I. K. Yap, S. Pani, N. T. Khor, S. F. Wong, R. Rosli, S. K. Cheong and C. O. Leong (2015). "Mutant p53-R273H mediates cancer cell survival and anoikis resistance through AKT-dependent suppression of BCL2-modifying factor (BMF)." Cell Death Dis **6**: e1826.

Thilakarathna, S. H. and H. P. Rupasinghe (2013). "Flavonoid bioavailability and attempts for bioavailability enhancement." Nutrients **5**(9): 3367-3387.

Vogiatzoglou, A., A. A. Mulligan, M. A. Lentjes, R. N. Luben, J. P. Spencer, H. Schroeter, K. T. Khaw and G. G. Kuhnle (2015). "Flavonoid intake in European adults (18 to 64 years)." PLoS One **10**(5): e0128132.

Wang, B. and X. H. Zhao (2017). "Apigenin induces both intrinsic and

extrinsic pathways of apoptosis in human colon carcinoma HCT-116 cells." Oncol Rep **37**(2): 1132-1140.

Wang, W., L. Heideman, C. S. Chung, J. C. Pelling, K. J. Koehler and D. F. Birt (2000). "Cell-cycle arrest at G2/M and growth inhibition by apigenin in human colon carcinoma cell lines." Mol Carcinog **28**(2): 102-110.

Wawryk-Gawda, E., P. Chylinska-Wrzos, M. Lis-Sochocka, K. Chlapek, K. Bulak, M. Jedrych and B. Jodlowska-Jedrych (2014). "P53 protein in proliferation, repair and apoptosis of cells." Protoplasma **251**(3): 525-533.

White, E., C. Karp, A. M. Strohecker, Y. Guo and R. Mathew (2010). "Role of autophagy in suppression of inflammation and cancer." Curr Opin Cell Biol **22**(2): 212-217.

Wong, R. S. (2011). "Apoptosis in cancer: from pathogenesis to treatment." J Exp Clin Cancer Res **30**: 87.

Xu, M., S. Wang, Y. U. Song, J. Yao, K. Huang and X. Zhu (2016). "Apigenin suppresses colorectal cancer cell proliferation, migration and invasion via inhibition of the Wnt/beta-catenin signaling pathway." Oncol Lett **11**(5): 3075-3080.

Zheleva, D. I., D. P. Lane and P. M. Fischer (2003). "The p53-Mdm2 pathway: targets for the development of new anticancer therapeutics." Mini Rev Med Chem **3**(3): 257-270.

Zhong, Y., C. Krisanapun, S. H. Lee, T. Nualsanit, C. Sams, P. Peungvicha and S. J. Baek (2010). "Molecular targets of apigenin in colorectal cancer cells: involvement of p21, NAG-1 and p53." Eur J Cancer **46**(18): 3365-3374.

국문초록

대장암세포에서 아피제닌과 루테올린이 p53 의존적 세포사멸과 자가포식에 미치는 영향

서울대학교 대학원 식품영양학과

유호수

플라보노이드 중 하나인 아피제닌과 루테올린은 구조적으로 매우 유사한데 아피제닌은 C링의 3번탄소에 OH기가 없으나 루테올린은 해당위치에 OH기가 존재한다는 차이점이 있다. 이러한 차이는 항산화 효과 등, 특정 기전 조절 여부 및 효능에 차이를 나타낼 수 있다. 아피제닌과 루테올린은 다양한 암종에서 세포사멸 및 세포분열을 조절함으로써 항암효과를 나타내는 것으로 알려져 있다. 최근 자가포식은 플라보노이드 계열이 가지는 항암효과 중 하나로서 보고되고 있으나 아피제닌과 루테올린에 의한 자가포식의 조절과 관련된 연구는 미흡한 실정이다. 따라서 우리는 대장암에서 구조적으로 유사한 아피제닌과 루테올린이 p53에 의존적인 방법을 통해 세포사멸 및 자가포식을 유도하여 항암효과를 나타내고 있는지를 확인하고자 하였다. 정상 대장세포에는 영향이 없는 농도 범위 내에서의 항암효과를 확인하고자 정상 대장 세포인 CCD-18CO와 p53 야생형 대장암세포인 HCT-116, p53 돌연변이 대자암세포인 HT-29 세포주를 사용하였다. 또한 p53에 의존적인 항암기전을 확인하고자 HCT-116과 HT-29 세포에서 단백질,

유전자 수준에서 관련 지표를 측정하였다. 그 결과, 아피제닌은 12.5-25 uM에서, 루테올린은 10, 20uM에서 처리농도가 증가함에 따라 p53 야생형 암세포인 HCT-116의 생존율을 감소시켰으나, p53 돌연변이 암세포인 HT-29에서는 저항성을 나타내었고 일반세포 CCD-18CO에서는 영향이 없었다. 특이하게도, 저농도의 아피제닌 0-5uM는 p53 돌연변이 세포의 생존율을 처리 농도에 따라 감소시켰다. 아피제닌과 루테올린이 p53 야생형 암세포에서 p53의 인산화 및 활성화하는 것을 확인하였고, 이를 통해 세포사멸 유도와 세포분열 저하를 일으킴을 확인하였다. 또한 아피제닌과 루테올린은 세포사멸 유도에 영향을 줄 수 있는 소포체 스트레스가 p53에 의존적인 방법으로 유발됨을 관찰하여 아피제닌과 루테올린에 의해 유발된 p53에 의존적 세포사멸이 p53 의존적으로 유발된 소포체 스트레스에 기인할 가능성이 있음을 확인하였다. 마지막으로, 아피제닌과 루테올린이 p53 야생형 암세포에서만 자가포식을 유도하는 것을 확인하였다. 그러나 오토파고좀과 라이소좀의 융합 단계에서 자가포식의 저해를 일으키는 Chloroquine을 처리하였을 때 아피제닌과 루테올린이 가지고 있던 세포사멸의 효과를 증가시키지 못하였다. 즉, 아피제닌과 루테올린이 유도한 자가포식을 저해하였을 때 두가지 물질에 의해 유발되던 세포사멸 및 생존율 저하가 저해되지 않음을 확인하였다. 결론적으로 이 연구에서 아피제닌과 루테올린이 p53에 의존적인 방식으로 세포사멸과, 자가포식을 유도하여 암세포의 성장을 저해한다고 할 수 있다.

주요어: 대장암, 아피제닌, 루테올린, p53, 자가포식, 세포사멸

학번: 2017-22435