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# The role of TNFα/p53 pathway in endometrial cancer mouse model administered with apple seed extract

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**Summary.** Recent studies regarding the ability to relieve and reconstitute the endometrium in the treatment of endometrial cancer are limited. In this study, to analyze endometrial cancer, early endometrial cancer was induced by injecting a colon cancer cell line into the lower abdominal cavity of mice. Subsequently, the apple seed extract was administered orally to determine if the extract could affect the endometrial cancer. Administration of apple seed extract to the endometrial cancer model confirmed that the apoptosis suppressing mechanism was downregulated concurrently with the reduced expression of NF-κB. In contrast, the TNFα/p53 pathway upregulated the apoptosis. A number of clinical inferences could be derived from the results of this study; moreover, the administration of apple seed extract in a cancer metastasis model has not been reported in earlier toxicity induction studies. The results of this study indicated that the apple seed extract partially enhances apoptosis and the immune function related factors in endometrial cells. By improving tissue remodeling, the extract may help to restore the endometrium.

**Key words:** Apoptosis, TNFα/p53, Apple seed, Endometrial Cancer, Uterus

## Introduction

The research on endometrial cancer is vital in women's cancer. Women undergo a lot of uterine associated stress during menstruation, pregnancy, and childbirth. Due to the uterus undergoing many changes, hormonal and physiological abnormalities are likely to occur and cause the development of cancers with a very high recurrence. Endometrial cancer (EC), which forms in the endometrium, accounts for 4.8% of all cancers worldwide and is classified as the most common cancer in developed countries. In particular, endometrial cancer may be caused by endometrial inflammation during menstrual cycles, and problems may develop due to dysregulation of physiological hormone mechanisms (Siegel et al., 2015; Morice et al., 2016). Another cause of endometrial cancer is cancer that accompanies colorectal cancer, which is classified as having the highest metastatic rate (approximately 9-19%), which is tenfold more dangerous in the average person and often occurs in young women (Yoon et al., 2005). Surgical methods are the preferred primary treatment of endometrial cancers, and it is anticipated that in future, conservative treatment will be used along with progestin therapy to increase preservation (Park et al., 2006). However, the risk of metastasis and recurrence at other organs may increase when conservative treatment is used without complete surgical treatment of the endometrium (Creasman et al., 1987; Gotlieb et al., 2003). It is also considered that cancer recurrence could increase after surgery. We have looked at several anticancer alternatives which focused on apple seeds (AP). According to Keddy et al. (2012), apple peel flavonoid fraction 4 (AF4) extracted from apples contains a number of polyphenol compounds including flavonols, anthocyanins, dihydrochalcones, phenolic acids, and flavan-3-ols such as flavonoids-rich extracts. Quercetin glycosides (quercetin-3-O-galactoside, quercetin-3-O-rutinoside, quercetin-3-O-glucoside, and quercetin-3-O-rhamnoside) constitute about 70% of the phenol content of AF4, which has shown neuroprotective and anti-inflammatory properties in cancer mouse models (Keddy et al., 2012; Warford et al., 2014; Ribeiro et al., 2015). In particular, in the results of Sudan and Rupasinghe (2014), AF4 not only induced cell cycle arrest and apoptosis but also inhibited the growth of hepatocellular carcinoma (HepG2) cells by acting as a topoisomerase toxicant (Sudan and Rupasinghe, 2014). The fruit seed contains Brucea javanica oil (BJO) (Yang et al., 2015), which includes oleic acid, linoleic acid, stearic acid, palmitic acid, arachidonic acid, and other unsaturated fatty acids (Ma et al., 2013). As one of the Chinese patented medicines, it has been widely used for the treatment of lung cancer (Nie et al., 2012); however, insufficient research has been done on its wider use. In the present study, we analyzed apple seeds' cytotoxic,

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antiproliferative, antimigratory, and tumor suppressive properties in an endometrial cancer model.

## Materials and methods

### *Animal dissection and ethical approval*

The experiment was carried out in accordance with the 2009 Korean Council on Animal Care guidelines (Lee, 2009). The dissection was carried out at the Hankyung National University experimental animal's anatomy laboratory with temperature, humidity and ventilation facilities. All the mice fasted the day before dissection, were sacrificed by the cervical vertebral dislocation method, and organs were then extracted. All animal procedures followed the protocol approved by the Animal Experimentation Ethics Committee at Hankyong National University (permission number: 2019-1). Ethics approval for animal use was obtained from the Hankyung National University Committee on Laboratory Animals and was in accordance with the Korean Council on Animal Care guidelines.

### *Preparation and certification of animals*

Female ICR mice (Institute of Cancer Research; 9-week-old female mice) were obtained from Dahan Bio Link (Eumseong, Korea) and maintained in light-controlled and air-conditioned rooms. Animals were kept on a 12 h dark/night schedule at a constant temperature of 25°C and 50% relative humidity. Cages (Type III: 420x260x150 mm) were prepared for the period during which mice were individually housed. Note: Data were collected for housing cage activity, food intake, and fecal corticosteroid metabolites (FCM). All surgeries were performed under pentobarbital sodium (50 mg/kg) anesthesia, and every effort was made to minimize pain. All ovaries were treated to stimulate ovulation and then used in the experiment. Stimulation involved injecting 5 IU PMSG (Serotropin; Teikoku Zoki, Tokyo, Japan) into the abdominal cavity and injecting 5 IU HCG (Puberogen®; Sankyo, Tokyo, Japan) 48 hours later.

### *Apple seed extract*

Apple seed (AS, apple variety: Malus pumila Mill (Jonathan), Korea) in ethanol was filter-sterilized and stored at -80°C. Prior to use in this study, ethanol was evaporated under nitrogen gas and the AS residue was dissolved in water without sterile pyrogen, and an aliquot of the resulting AS liquid stock (10 mg/mL) was stored at -20°C.

### *Animals and HCT116 cancer cell-line implantation procedure; AP treatment*

The cell line was injected to construct an early endometrial cancer model that metastasized from colorectal cancer. The uterus position of the left lower abdomen of the centerline was marked for injection (skin and muscles). $1x10^6$ of HCT116 (ATCC, Manassas, VA, USA) cells suspended in 50 µl of sterile water for injection (BITD ruginfo, SC, Kor) were injected twice every seven days directly under the skin where the uterus is located (Kim et al., 2020). A 0.3 mm insulin syringe (Omnican 50, B-Braun, Melsungen, Germany) was used for the injection. After being injected, the animals were placed in a warm environment, and changes to their bodies were observed. Oral administration of AS at 100 mg (based on 30 g) in 200 ml drinking water (Endometrial cancer group: five mice, Normal group: five mice) or drinking water (Endometrial cancer group: five mice, Normal group: five mice) were administered every other day for 13 days. Mice were monitored daily, and their physical activity and body weights were recorded. Mice were euthanized on day 14, after which they were dissected, and their uterus was excised and photographed. Mice from all experimental groups were subjected to blood sampling from the heart on the day of dissection. In addition to a complete blood count, serum levels of albumin were measured on the same day in every study subject. For the serum extraction method, extracted whole blood was centrifuged at 4000 RPM at room temperature for 10 minutes to separate only the serum, and then used for the experiment.

### *Histological analysis of the endometrium*

The uterus was fixed in 70% Diethylpyrocarbonate (DEPC)-ethanol, embedded in paraffin, and cut into 5 um-thick sections. Uterus sections were mounted onto glass slides and stained with hematoxylin and eosin for the detection of necrotic and live cells. Additionally, uterus tissue was stained with Alizarin Red solution (Sigma-Aldrich) for 30 min; staining was performed for histological inspection with an optical microscope (X40, X100, and X400).

### *Behavioral Assessment of Stress*

The behavioral assessment of mice in each treatment group was performed according to the research results of Lezak et al. (2017), and Luo et al. (2020).

Mouse activity analyzed was evaluated from 1 point to 10 points depending on the intensity of the response to external stimuli. The mild stress assessment methods for predicting the activity of all mice were restraint, lightly squeezing the tail, horizontal shaking, and slanting the cage.

10 min measurements, Criteria for stress evaluation: The highest score is rated when each treatment group responds or struggles sensitively to each stress relative to the normal mice response. On the contrary, if there was no or little reaction of the mice, the lowest score was given.

Management evaluation of all experimental animals was conducted according to the Korean IACUC Guidelines (Lee, 2009).

### Hormone ELISA

For quantification of a specific protein from the uterus protein, a primary antibody (LH (8G9A2 Abcam, Cambridge, UK), FSH (sc-7797 Santa Cruz Biotechnology Inc., Texas USA), IGF (sc-712 Santa Cruz Biotechnology Inc., Texas USA), and 20a-HSD (ab192785 Abcam, Cambridge, UK)) was applied to a 96-well ELISA plate at 4°C for one day. The plate was washed twice using washing buffer (1X PBS with 2.5% Triton X-100) and blocked using 1% skim milk blocking solution at 4°C for 24 h. After washes with the washing buffer, immune reactions were detected using secondary antibodies (HRP-conjugated anti-goat (ab6721-1, Abcam, Cambridge, UK) or anti-mouse (ab6741, Abcam, Cambridge, UK) secondary antibodies) for 2 h. A substrate solution (R&D Systems, USA) was added for the reaction. To stop the reaction, 1 M NH2SO4 was used, and absorbency was measured at 450 nm.

### Quantification Real-Time PCR

Total RNA, extracted from the uterus tissue scrapings using TRIzol reagent (Invitrogen, CA, USA), was treated with DNAse (Ambion, Austin, TX), as per the manufacturer's instructions, and quantified by UV spectrophotometry. First-strand cDNA was synthesized by reverse transcription of mRNA using Oligo (dT) primer and SuperScript II Reverse Transcriptase (Invitrogen, Grand Island, NY). For real-time RT-PCR, the Line-gene K (Bioer Technology, Tokyo, JPN) was used to make a final reaction volume of 25 $\mu$l, with SYBR Green (TOYOBO, Osaka, JPN). The primers listed in Table 1 were used for the PCR, and the amplification completed under the following conditions: 10 min at 94°C, followed by 39 cycles of 30 sec at 94°C, 30 sec at 60°C or 65°C, 55 sec at 72°C, and a final extension for 5 min at 72°C. Rotor-Gene Real-Time Software 6.0 was used to analyze the cycle threshold (Ct) and to obtain a semi-log amplification plot. Finally, the relative expression levels of each gene were calculated using the $2^{-\Delta\Delta Ct}$ method by normalization with porcine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels.

### Western blot

Uterus tissue protein extracts (30 $\mu$g) were separated

**Table 1.** Primers for qRT-PCR.

| Gene | Type | Sequence | Gene | Type | Sequence |
|---|---|---|---|---|---|
| FAS | F.W | 5'-CCTGCATGGCAGTTACACAC-3' | BAX | F.W | 5'-GTGAGCGGCTGCTTGTCT-3' |
| | R.V | 5'-TTGCAAGACATGTCGGAAAG-3' | | R.V | 5'-GGTCCCGAAGTAGGAGAGGA-3' |
| P53 | F.W | 5'-ACAGTCGGATATCAGCCTCG-3' | IGF-1 | F.W | 5'-TGTCGTCTTCACATCTCTTCTACCTG-3' |
| | R.V | 5'-TTTTTTGAGAAGGGACAAAAGATG-3' | | R.V | 5'-CCACACACGAACTGAAGAGCGT-3' |
| BCL-2 | F.W | 5'-CAGGTATGCACCCAGAGTGA-3' | TNF-α | F.W | 5'-CCACCACGCTCTTCTGTCTAC-3' |
| | R.V | 5'-GTCTCTGAAGACGCTGCTCA-3' | | R.V | 5'-AGGGTCTGGGCCATAGAACT-3' |
| Casp-3 | F.W | 5'-CCTCAGAGAGACATTCATGG-3' | VEGF | F.W | 5'-TTACTGCTGTACCTCCACC-3' |
| | R.V | 5'-GCAGTAGTCGCCTCTGAAGA-3' | | R.V | 5'-ACAGGACGGCTTGAAGATG-3' |
| Casp-8 | F.W | 5'-GCAGAAAGTCTGCCTCATCC-3' | IL-2 | F.W | 5'-AACCTGAAACTCCCCAGGAT-3' |
| | R.V | 5'-GGCCTCCATCTATGACCTGA-3' | | R.V | 5'-CGCAGAGGTCCAAGTTCATC-3' |
| Casp-9 | F.W | 5'-AGTTCCCGGGTGCTGTCTAT-3' | IL-8 | F.W | 5'-ATGGCTGCTCAAGGCTGGTC-3' |
| | R.V | 5'-GCCATGGTCTTTCTGCTCAC-3' | | R.V | 5'-AGGCTTTTCATGCTCAACACTAT-3' |
| Cyt C | F.W | 5'-GAGGCAAGCATAAGACTGGA-3' | Bcl-XL | F.W | 5'-AACATCCCAGCTTCACATAACCCC-3' |
| | R.V | 5'-TACTCCATCAGGGTATCCTC-3' | | R.V | 5'-GCGACCCCAGTTTACTCCATCC-3' |
| ATG 5 | F.W | 5'-GGACCTTCTACACTGTCCATCC-3' | c-FLIP | F.W | 5'-TCCAGAATGGGCGAAGTAAAGAGC-3' |
| | R.V | 5'-TGTCATTCTGCAGTCCCATC-3' | | R.V | 5'-AGTCTCTTCACGGATGTGCGGAG-3' |
| MAP1LC3 | F.W | 5'-GATAATCAGACGGCGCTTGC-3' | NF-kB | F.W | 5'-ACCACTGCTCAGGTCCACTGTC-3' |
| | R.V | 5'-ACTTCGGAGATGGGAGTGGA-3' | | R.V | 5'-GCTGTCACTATCCCGGAGTTCA-3' |
| mTOR | F.W | 5'-CTGGGACTCAAATGTGTGCAGTTC-3' | IGFBP3 | F.W | 5'-AAGCACCTACCTCCCCTCCCAA-3' |
| | R.V | 5'-GAACAATAGGGTGAATGATCCGGG-3' | | R.V | 5'-TGCTGGGGACAACCTGGCTTTC-3' |
| PI3K | F.W | 5'-AGGAGCGGTACAGCAAAGAA-3' | ATG 12 | F.W | 5'-GGCCTCGGAACAGTTGTTTA-3' |
| | R.V | 5'-GCCGAACACCTTTTTGAGTC-3' | | R.V | 5'-CAGCACCGAAATGTCTCTGA-3' |
| AKT-r | F.W | 5'-TGAAAACCTTCTGTGGGACC-3' | ATG 16L1 | F.W | 5'-TGGCTGGAGTGCGATCTTCC-3' |
| | R.V | 5'-TGGTCCTGGTTGTAGAAGGG-3' | | R.V | 5'-CAGACGGCAAACGACTGTCCT-3' |
| PDK1 | F.W | 5'-CCGGGCCAGGTGGACTTC-3' | ATG 13 | F.W | 5'-TGGCGGAAGATTTGGACTCC-3' |
| | R.V | 5'-GCAATCTTGTCGCAGAAACATAAA-3' | | R.V | 5'-GGGTTTCCACAAAGGCATCG-3' |
| PKA | F.W | 5'-CAGGAAAGCGCTCCAGATAC-3' | CDK28 | F.W | 5'-GACACTCAGGCTGCTGTTCT-3' |
| | R.V | 5'-AAGGGAAGGTTGGCGTTACT-3' | | R.V | 5'-AGGTAAGGCGGAGGGTACAT-3' |
| BAD | F.W | 5'-GCCCTAGGCTTGAGGAAGTC-3' | CD3e | F.W | 5'-GCAGGCAAAGGGGACAAAAC-3' |
| | R.V | 5'-CAAACTCTGGGATCTGGAACA-3' | | R.V | 5'-CAGGCCAGAATACAGGTCCC-3' |

using 13% sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA). The membranes were blocked overnight with 5% non-fat dry milk at 4°C, washed for 10 min with washing buffer (0.1% v/v Tween20, 50 mM Tris-HCl [pH 7.6], 200 mM NaCl), and incubated for 2 h with specific antibodies. The primary antibodies used in this study were primary antibody (β-actin, TNF-r (each sc-47778; sc-374186 Santa Cruz Biotechnology Inc., Texas USA), P53 (PA5-27822 Invitrogen Grand Island, NY), Casp-3 (ab13847 Abcam, Cambridge, UK), IL-2 (ab106016 Abcam, Cambridge, UK), TIMP-2, TIMP-3 (each sc-21735; sc-373839 Santa Cruz Biotechnology Inc., Texas USA). After antibody binding, the membranes were washed three times for 15 min each with TBS-T buffer and then incubated for 2 h with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (diluted 1:5000). The membranes were incubated in the dark for 5 min in ECL detection reagent and then exposed to a sheet of diagnostic film in a film cassette for 1-30 min. Blood serum was then electrophoresed in 13% sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to evaluate protein profile.

### *Gelatin-Zymography*

20 ug of total protein was added to 10 $\mu$L FOZ loading buffer (5% Bromo phenol blue, 10% SDS, and 2% Glycerol) to analyze the MMP enzyme reaction from mice uterus tissues. They were mixed and allowed to react on ice for 5 min, and then electrophoresed for 1 h 30 min at 150 V with a gelatin SDS-PAGE gel containing 100 mg/mL gelatin. After electrophoresis, the gel was induced twice in protein renaturation with renaturation buffer (2.5% Triton X-100, 1X PBS) for 20 min and then washed with sterilized water for 20 min. The cells were placed in a zymography reaction buffer (1 M Tris-HCL pH 7.5, 5 M NaCl, 1 M $CaCl_2$, 0.2 mM $ZnCl_2$, 0.2% Triton X-100, 0.02% $NaN_3$) and then allowed to react at 37°C for 18 h. After completion of the reaction, the zymography gel induced protein was stained with Coomassie Brilliant Blue (Bio-rad, USA) for 1 h, after which the color faded and discolored parts were analyzed (Kim et al., 2018).

### *Tissue clearing for three-dimensional immune detection of the uterus*

The CUBIC (Tokyo Chemical Industry, T0781, Japan) experiment was performed as described in the Kagami et al. (2018) study (Fig. 1). The uteruses of the female mice were sacrificed and fixed with 4% paraformaldehyde (PFA) in PBS. Fixed organs were further immersed in 4% PFA at 4°C overnight and then incubated in CUBIC-1 reagent at 37°C for five days with gentle shaking. After washing three times using PBS with gentle shaking at room temperature for 30 min, the organs were immersed in 20% sucrose in PBS at 4°C for one day, and incubated in CUBIC-2 regent at room temperature for two days. The samples were blocked at RT (Room Temperature) for 1 h in TBS (1X PBS with 0.01% Triton X-100), containing 5% normal horse serum (NHS). The antigen-antibody reaction was induced using primary antibodies (Casp-3, IL-2, VEGF (PA5-16754 Invitrogen Grand Island, NY) and IgG (sc-516102 Santa Cruz Biotechnology Inc., Texas USA), diluted 1:200 in blocking buffer overnight at 4°C. The samples were then incubated with Alexa-488 or Alexa-594 (Molecular Probes) antibodies (diluted 1:500 in blocking buffer) for 2 h at RT and then washed with PBS for 30 min. The sample was finally dyed with DAPI and observed in the Confocal Laser Point Scanning Microscope (C2; Nikon Corp., Tokyo, Japan), and organizational analysis was performed with NIS-Elements C software (ver. 3.2).

### *Immunohistochemistry*

The paraffin slide of each uterus tissue was used to conduct IHC experiments. Deparaffin / hydrate was

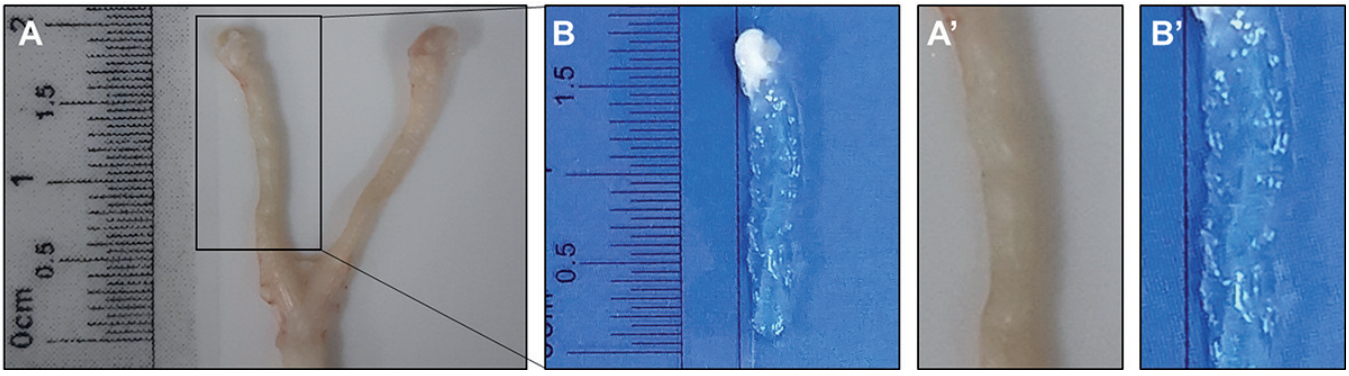


**Fig. 1.** The procedural protocol of tissue clearing of the mice uterus using CUBIC. **A.** Bright-field images of uterus of wild-type mice before. **B.** After tissue clearing. **A', B'.** A higher magnification image within the black square.

repeated twice using Xylene, 100% Ethanol, 95% Ethanol for 10 min, washing in ddW for 5 min, and boiling in 10 mM sodium citrate for 10 min. Antigen retrieval was performed by heating at 95°C in 10 mM sodium citrate (pH 6.0). Endogenous peroxidases were quenched with 0.3% hydrogen peroxide in methanol for 5 min at room temperature. After three washes in 1X PBS buffer, the slides were blocked in 1% goat serum containing 3% horse serum for 1 h at room temperature. The primary antibodies against (Casp-3) were detected overnight at 4°C. And the secondary antibody was detected at room temperature (25°C) for 1 hour. All sample was washed three times for 3 minutes with 1×PBS, and then 300 $\mu$l of reactive-substrate (3,3'diaminobenzidine, DAB) was added, and the chemical-reactive was allowed to induced for up to 10 minutes before washing with ddW. Afterward, all samples were counter-stained with Harris hematoxylin solution containing PAS (periodic acid-Schiff) reagent and 4% acetic acid. Tissues were dehydrated, cleared, and covered with Permount solution (Fisher, NJ, USA), and observed under a microscope (Nikon Corp., Tokyo, Japan) at 200X and 400X magnification. In addition, it was evaluated using an anti-Apoptotic detector to confirm apoptotic in the uterine tissue (Kim et al., 2012).

### *Statistical analysis*

ELISA and Real-time PCR data were subjected to T-test and GLM using Statistical Analysis System software (SAS Institute, version 9.4, Cary, NC, USA). Differences among mean values from each treatment group were determined using Duncan's multiple range tests. The statistical significance was established at $P<0.05$.

## Results

### *Anatomical and behavioral changes of mice according to the treatment group*

Table 2 and Fig. 2 show the results of changes in blood, behavioral, and anatomical parameters after human colon cancer was injected near the lower abdominal uterus. Through the analysis of blood changes among each treatment group, it was confirmed that the level of leukocytes increased in the cancer metastasis

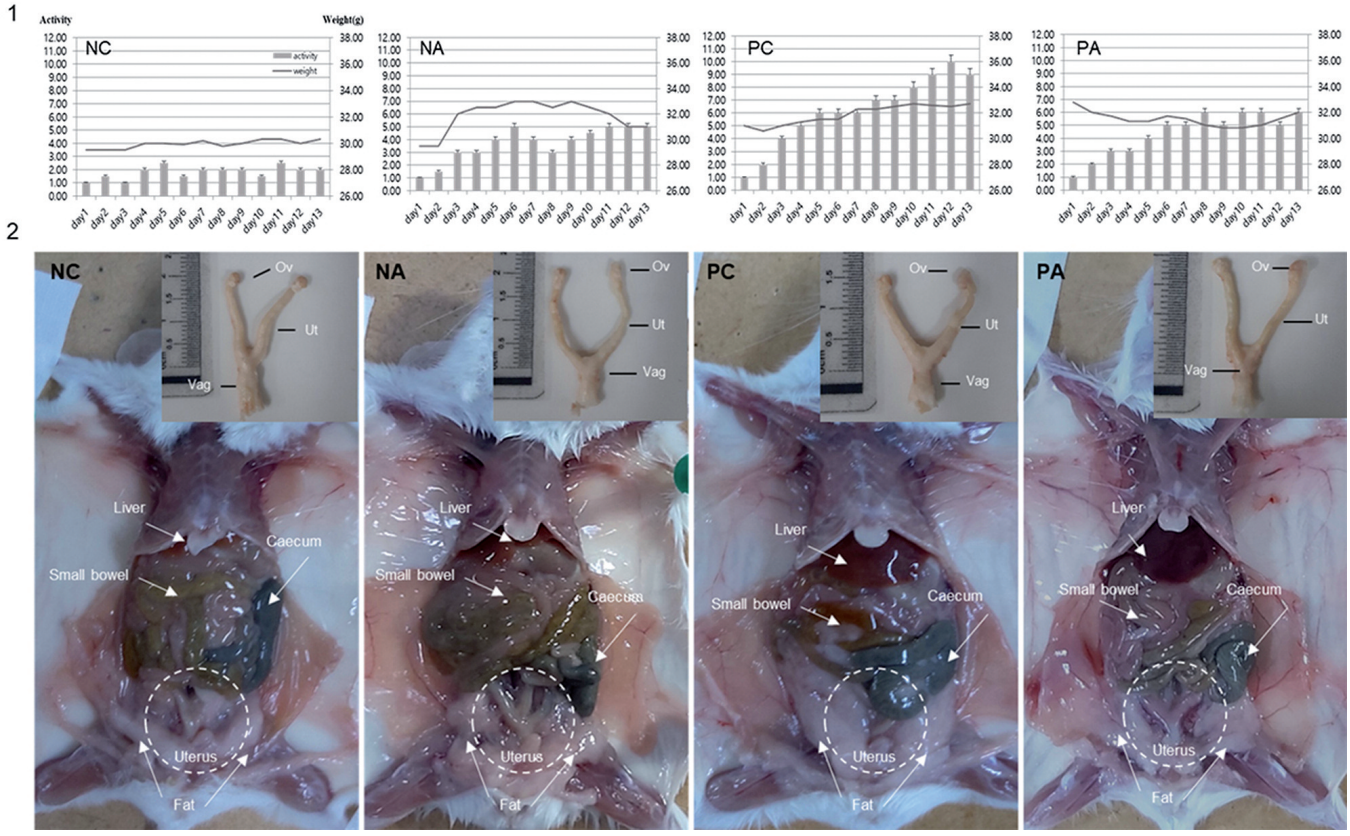


**Fig. 2.** Anatomical evaluation, behavioral patterns and changes in body weight in the mice representing each treatment group. **1.** Behavioral Assessment. Changes in behavior were measured and evaluated according to IACC standards. Five mice in each group were measured and expressed as the average. **2.** Anatomical evaluation. The dissection of the mice was cut to the center of the abdomen using a scalpel. The anatomical identification of each treatment group was evaluated by comparing the location, shape, color, distribution of fat, and shape of the digestive organs.

model group (PC) and the group treated with apple seeds extract, in comparison with the untreated control group (NC). In PC, the immunocyte count was the highest at 48.2±1.45%, and in the apple seed extract-treated group in the metastasis model group (PA), it was slightly reduced to 36.4±0.35% (Table 3). With regard to behavioral change, based on the comparison with NC, vast differences were observed between individuals. However, it was confirmed that the behavioral change was stabilized in the group treated with substances in the cancer metastasis model group (PA) (Fig. 2-1). Regarding anatomical changes, in PC, the accumulation of visceral fat was very high and lesions near the small intestine and large intestine were observed. The liver

**Table 2.** Count of red blood cells and white blood cells in the complete blood count of each group.

| Samples | Total blood cell rate | Cell type | | Immunocyte (%) |
|---|---|---|---|---|
| | | Red blood | White blood | |
| NC | 316±20.5 | 256±10.5** | 60±10.7 | 8.0±0.87 |
| NA | 335±20.3 | 215±12.4 | 120±7.7 | 22.5±0.77 |
| PC | 477±15.2 | 247±5.8* | 230±10.9** | 48.2±1.45** |
| PA | 362±16.5 | 230±6.7 | 132±10.5* | 36.4±0.35* |

Normal endometrium (NC: Normal control), endometrium of the normal group administered with apple seeds (NA: Normal group + apple seed), Endometrium of endometrial cancer group (PC: Endometrial cancer Positive control), Endometrium of endometrial cancer group administered with apple seeds (PA: Endometrial cancer Positive control + apple seed). *,**Different letters within the same column represent a significant difference ($p<0.05$).

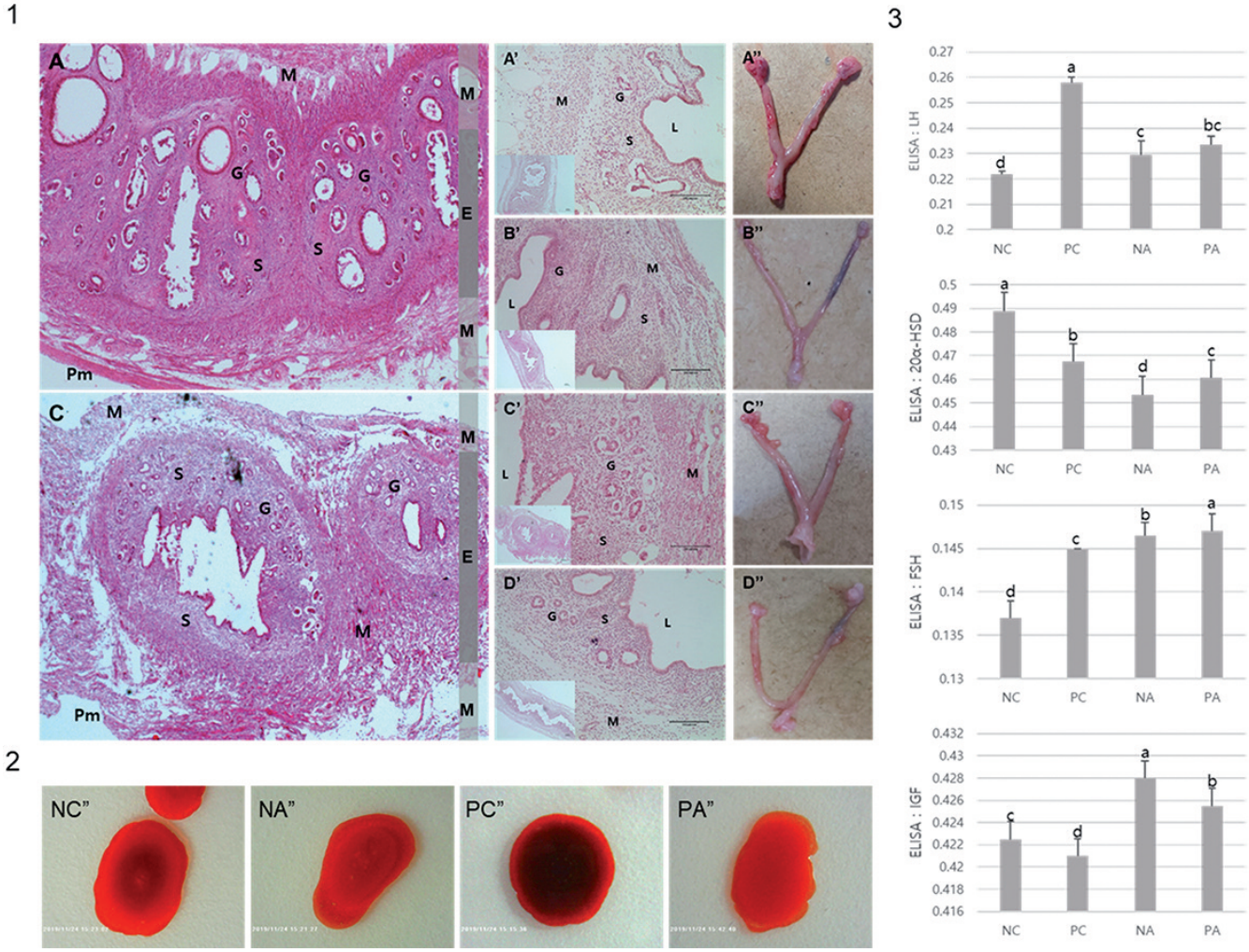


**Fig. 3.** Analysis of hormone levels and histological changes in the uterus in each treatment group. **1.** H & E staining for morphological analysis of mouse uterus in each treatment group. **A.** endometrium in normal group. **C.** uterus in endometrial cancer group. **A'-D'.** normal endometrium (A' : NC) and endometrium of the normal group administered with apple seeds (B' : NA), Endometrium of endometrial cancer group (C' : PC), Endometrium of endometrial cancer group administered with apple seeds (D' : PA). **A''-D''.** a photograph of the uterus extracted by dissection. G, glandular area; S, stromal cell zone; M, myometrium; L, lumen. **2.** Assessment of blood cruor formation. A drop of blood was dropped on the slide and evaluated in 3 min. **3.** ELISA analysis to assess hormone levels. ELISA experiments were repeated three times, and data are average fold change (mean ± SD). The levels of hormones were determined according to a standard curve, which takes into account four parameters based on the following equation: y = (A – D)/(1 + [x/C]^B) + D. a,b,c,d,e. Different letters within the same column represent a significant difference ($p<0.05$). Scale bars: 100 µm.

descended below the lower abdominal cavity, and it was difficult to observe the uterus. In contrast, the visibility of the small and large intestine was acceptable in PA, and it was confirmed that a decreased amount of fat accumulated near the uterus. Thus, it was confirmed that the fat accumulation rate in PA was reduced compared to PC. Although no traces of the tumor were observed, changes in organs (PA group) seemed to have been restored as in NC, and morphological features of the uterus too were similar to NC (Fig. 2-2).

### *Endometrial changes and hormone differences in each group*

Fig. 3 shows the results of analyzing the changes in the endometrium among NC, PC, and PA. As a result of confirming the hematologic phenomenon of each mouse group, other groups other than the PC group showed normal blood parameters. However, in PC significantly increased blood cruor phenomenon was observed, while PA showed similar blood parameters as in the normal group (Fig. 3-2). In NC, the homogeneity of the cells in the endometrial cell layer or/and the borders and cell compartments of myometrium and endometrium were uniform, and the material-treated group (NA) showed similar results. However, the distribution of the glandular cell zone was lower than that of the normal group. In PC and PA, the density and homogeneity of endometrial cells were relatively low compared to the normal group, while the lumen section was more expanded. Additionally, lower deposition of fat was observed in the glandular cells, connective tissue sections, and the cell density was also lowered. In PC, the shape of the tumor with dense cells in the endometrium, and the increase in inflammation in the epithelial tissue was confirmed. However, the endometrium of PA seemed to have recovered and appeared similar to that in NA. Further, the endometrium deposition rate, the fat section, and the cell detachment section were normally restored (Fig. 3-1). Comparative analysis of hormones showed differential profiles in each treatment group. In the case of NC, 20α-HSD increased, but FSH and LH hormones were lower than in other groups. PC had a relatively upregulated LH, while the expression of IGF2 was relatively low. In comparison, the group treated with the substance had increased FSH. Further, in the case of PC, LH and FSH hormones were simultaneously detected, whereas, in PA, only FSH increased (Fig. 3-3).

### *Comparison of expression patterns of immune factors and cell survival-related genes among treatment groups*

Analysis of the IL-2 and IgG expression (the immune factors) in the uterus of each treatment group showed that the expression of the IgG was higher than IL-2 in the endometrium of PC (Fig. 4-1). Further, the expression of both factors was low in the myometrium (Fig. 4-1). In contrast, both IgG and IL-2 were highly expressed in the endometrium and myometrium of PA, and the endometrial lesions (Fig. 2-1) increased in the identified sections. Thus, it was confirmed that compared to IgG, IL-2 was highly upregulated in PA. Comparison of the mRNA expression pattern in the different groups relative to NC showed that most of the factors related to the angiogenic pathway were increased in PC. NF-ϰb was increased in the PI3K mechanism system, but the IGF related to nutritional metabolism was downregulated, while the expression of the VEGF factor was highly upregulated. In comparison, GFBP3,

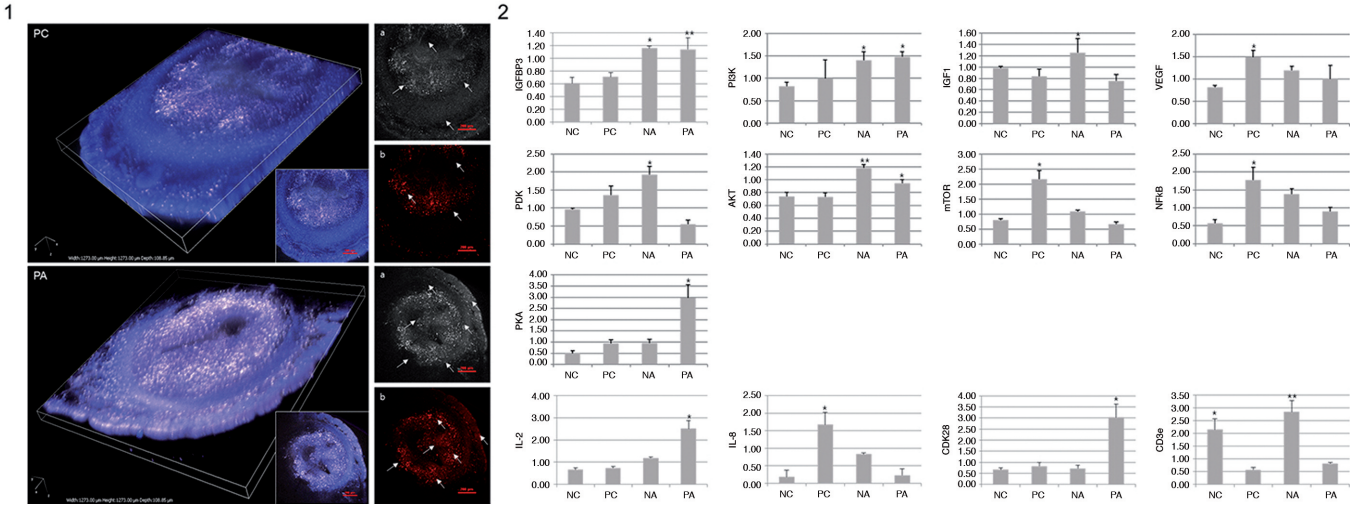


**Fig. 4.** Analysis of immune-related genes and survival signal-related genes during normal and endometrial cancer group. **1.** Three-dimensional and cross-sectional images of the uterus detected with IgG and IL-2 proteins. **A.** endometrial cancer group. **B.** endometrial cancer group administered with apple seeds, a) IgG protein detected, b) IL-2 protein detected. **2.** qRT-PCR analysis of survival signal-related genes in each group. Experiments were repeated three times, and data are expressed as mean ± standard error ($P<0.05$). Scale bars: 100 µm.

AKT, and PKA were relatively increased in PA. The expression profile of factors related to the immune system showed significantly higher levels of IL-2 and CDK28 in PA, while IL-8 was significantly higher in PC and other factors were relatively low in this group (Fig. 4-2).

### *Expression pattern of apoptosis factor among each treatment group*

The results of the comparative analysis of expression patterns of apoptosis-related factors in each treatment group are shown in Fig. 5. The results of the proteomic analysis in blood showed that PC increased from 53 kD to about 35 kD in the albumin position compared to the other groups, and showed high expression of proteins in the 150 kD and 90 kD regions (Fig. 5-5). In the case of apoptosis-related gene expression, the expression of factors related to the p53 signal pathway was increased in PA, and the inhibitor gene showed low expression. However, in the case of PC, most of the genes related to apoptosis were downregulated, while the expression of BCL-2 and XL was relatively high. These results displayed a similarity in the protein expression pattern (Fig. 4-4), and the upregulated expression of TNF-r, p53, and Casp-3 in PA; notably, the expression of active-Casp-3 was upregulated in PA. In comparison, the expression of TNF-α was relatively low in PC, while that of p53 was not different from the normal group. However, the expression of active-Casp-3 was higher than observed in NC and NA (Fig. 5-3). Regarding the analysis of the MMPs activity related to the change of cell-substrate, negligible activity of MMP-9 was observed in PC, while the activity of MMP-2 was relatively high in PA. The expression patterns of TIMPs that inhibit MMPs showed very low expression compared to the MMPs activity (Fig. 5-6). In particular, the expression of TIMPs was confirmed in NC and PC, but not in NA. However, in the case of PA, the expression of TIMPs was slightly increased and related to the expression pattern of MMPs (Fig. 5-4). Further, the expression of Casp-3 showed a relatively high mRNA expression pattern in both the inner and outer membranes of the uterus of PA (Fig. 5-1,5-2); the

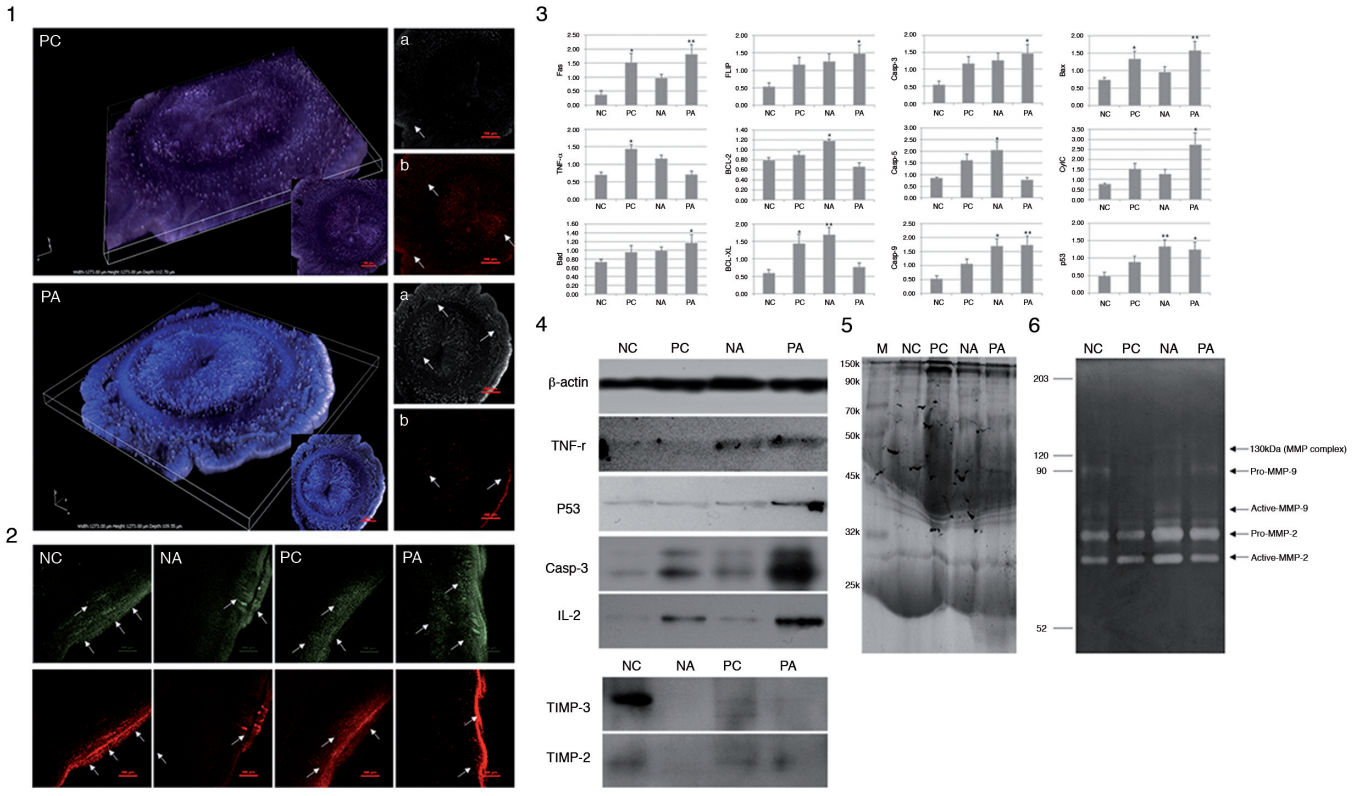


**Fig. 5.** Expression evaluation of apoptosis and extracellular matrix associated genes in endometrium section of each group. **1.** Three-dimensional and cross-sectional images of the uterus detected with Casp-3 and VEGF proteins. **A.** Endometrial cancer group. **B.** Endometrial cancer group administered with apple seeds, a) Casp-3 protein detected, b) VEGF protein detected. **2.** Immunohisto-fluorescence staining of myometrium section showed that the Casp-3 and VEGF protein were detected in each group. **3.** qRT-PCR analysis of apoptosis signal-related genes in each group. **4.** Western blot analysis in each group. Data represent the mean ± SEM of five individual experiments and were normalized against β-actin (Housekeeping gene) as an internal standard. **5.** Analysis of differences in serum proteins in each group. **6.** Zymography analysis of the MMPs activity in endometrium proteins of each group. Scale bars: 100 μm.

expression in the outer membrane was especially very high. It was confirmed that the expression of VEGF protein was very high in both the inner membrane and the outer membrane in PC, and in particular, it was relatively high in the section of the blood vessels of the outer membrane, as shown in Fig. 5-2. In contrast, PA showed a lower expression than PC and the expression was confirmed in the outer membrane, but the expression in the inner membrane was very low.

*Analysis of expression patterns of apoptotic factors in endometrium among each treatment group*

Analysis of the expression pattern of apoptotic factors in the endometrium of each group showed that the expression of these factors was very low in PC as shown in Fig. 6-1, 6-2. In the case of PA, the expression of Casp-3 increased in the endometrial epithelium, and the expression of apoptotic factors showed a similar pattern. Results of the analysis of the change of Ca+ ion that examined the calcification of the endometrium and the perimetrium showed that progression of calcification could not be confirmed in the uterus. Although the response was not observed in NC and NA, PC showed a very high response in the endometrium. However, in the case of PA, the red response in the endometrium was reduced, and it was confirmed that the glandular zone, which showed the highest response in the PC, was very

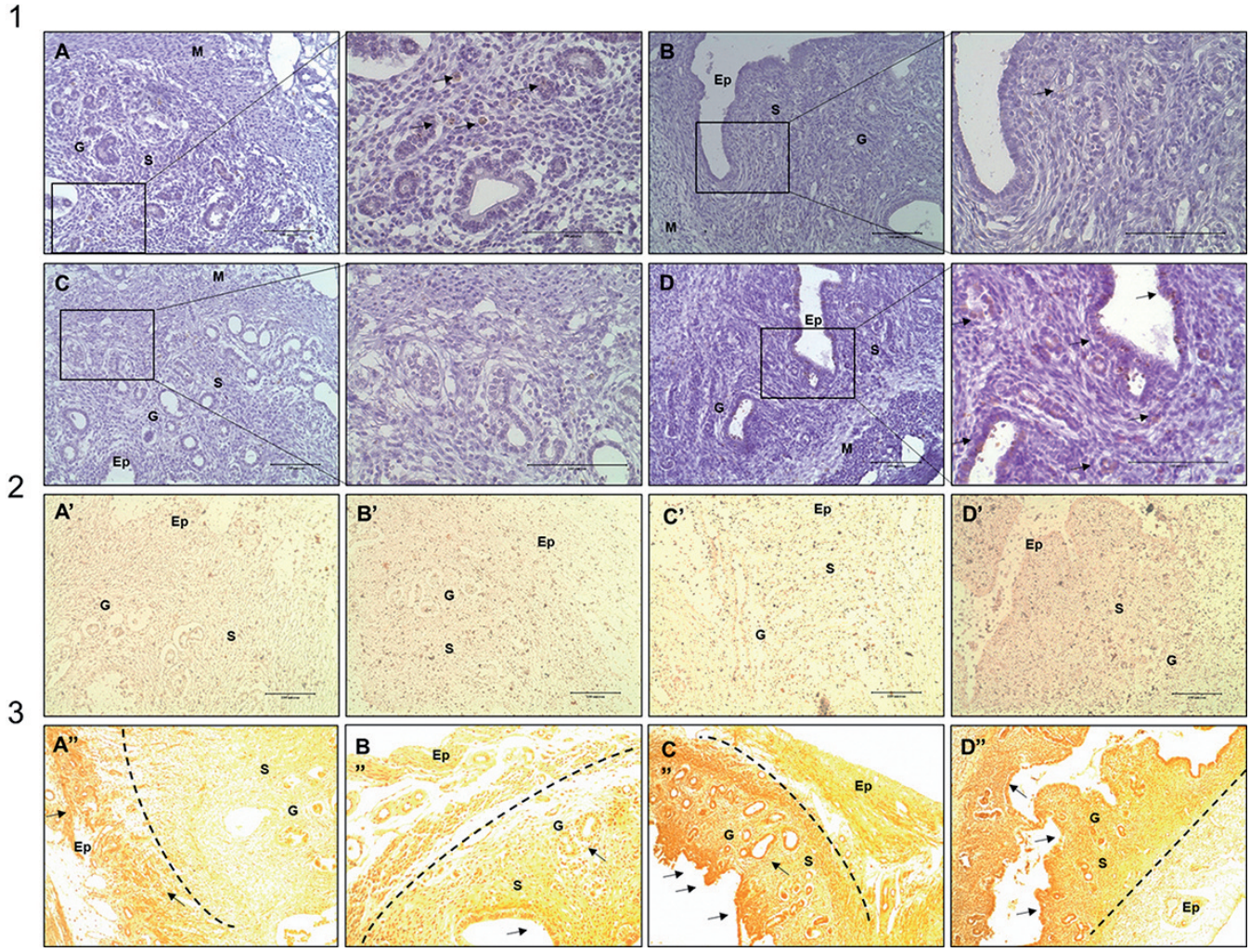


**Fig. 6.** Expression and localization of Casp-3 and apoptotic label during endometrium tissues in each group. **1.** Tissue sections of endometrium were immune-stained with the Casp-3 antibody and counterstained with Hematoxylin. Black arrows indicate Casp-3-expressing cells. **A.** Normal group. **B.** Endometrium of the normal group administered with apple seeds. **C.** Endometrial cancer group. **D.** Endometrium of the endometrial cancer group administered with apple seeds. **2.** Apoptosis detection analyses used terminal deoxynucleotidyl transferase to label 3'-OH ends of DNA fragments that were generated during the process of apoptosis. Detection method used Anti-FITC HRP Conjugate (1:200) and DAB kit. The dark brown section is where apoptosis is detected. **3.** Alizarin Red staining. The reaction of Alizarin Red in the endometrium of the PC increased very highly and decreased in PA. G, glandular area; S, stromal cell zone; Ep, Epithelial cell point; M, myometrium. Scale bars: 100 μm.

low in PA (Fig. 6-3).

## Discussion

According to a study by Yadegarynia et al. (2012), the compounds derived from vegetables have toxic effects during the metabolism of cancer cells but do not affect the metabolism of normal cells14. Further, according to a study by Loung et al. (2019), AF4 from apple peels inhibited the development of cancer via suppressing estrogen receptors. The study also found that apples affected the metabolic process of cancer cells. Based on these results, we speculated that apple seeds may have a deterrent effect on cancer. However, apple seeds contain a significant amount of toxin amygdalin (1.0–3.9 mg/g seeds), which is classified as a mandelonitrile gentiobioside called cyanogenic glycoside (CG) that can be hydrolyzed to toxic hydrogen cyanide in mammals (Zagrobelny et al., 2004; Haque and Bradbury, 2020). Thus, this specific property of the compound may affect cancer cells (Miura et al., 2007). However, studies on whether apple seeds may act against endometrial cancer are lacking. Another important point for endometrial cancer is that it has a deep relationship with the action of the apoptosis factor; it is related to the expression of PKC and affects the regulation of apoptosis (Haughian et al., 2006). We sought to analyze the effect of apple seed extract on endometrial cancer. In the present study, we injected a colon cancer cell line, and metastasis and induction of endometrial cancer was analyzed. Next, we explored whether administration of apple seed extract for a certain period could affect early endometrial cancer. We sought answers to two questions. First, can the administration of apple seeds affect the endometrium that has metastatic endometrial cancer? Second, can the apple seed extract induce the apoptosis of endometrial cancer? In previous studies, we have shown that colorectal cancer cell lines affect the uterus and spread to endometrial cancer (Kim et al., 2020), and anatomical findings showed abnormalities in the digestive tract and changes in the endometrium similar to previous results. The evaluation of mRNA expression showed that the expression of NF-ϰB, BCL-XL, and BCL-2 involved in the PI3K/AKT pathway was increased in the endometrial cancer-induced group, while the expression of factors related to the mechanism of internal/external apoptosis was suppressed (Uddin et al., 2010). Additionally, it was confirmed that the expression of VEGF protein was increased in the endometrium and myometrium. These results confirmed that VEGF expression was gradually increased in the endometrial cancer-induced group, and the results were in line with those reported by Sillars-Hardebol et al. (2010), and increased through the angiogenic part of the endometrium and the distribution of capillaries. It has been shown the incidence of VEGF and cancer progression are closely related (Otrock et al., 2007; Roskoski, 2007). In our study, the apple seed administration led to suppression of VEGF expression. Further, an increase in MMP-9 expression leads to histological changes in the endometrium and the glandular area of the endometrium is shown to be restored (Warren, 2008; Contreras et al., 2010). In addition, the results confirmed that IL-2 expression was increased in the endometrium of the substance-administered group, as shown by Abdel-Hamid et al. (2019). However, in the endometrial cells of the endometrial cancer-induced group, IgG showed a higher expression than IL-2, which can be attributed to the autoreverse action in the cancer-forming section, as shown by Xu et al. (2018). Therefore, our results of administering apple seeds in the endometrial cancer model confirmed that it inhibited the apoptosis suppressing pathway as seen by the reduced expression of NF-ϰB. Further, it was confirmed that TNFα and p53 increased the apoptosis mechanism. In particular, the expression of Casp-3 was shown to be increased in the epithelial cells of the endometrium. In other words, the use of apple seeds seems to improve the function of the p53 pathway in endometrial cancer. These results can be attributive to the specific action of cyanide (Guo et al., 2013; Haque and Bradbury, 2020), which is a component of an apple seed, but it is unlikely to be due to the action of cyanide alone. Several studies have shown that amygdalin, which comes from apple seeds, acts as a toxic agent and can cause serious problems in living organisms. However, many people eat apple seeds, and the chances of cyanide toxicity due to occasional ingestion of apple seeds are very low. Therefore, this study administered the whole extract of apple seeds without purification of amygdalin, and the extract did not show any adverse effects in the normal group as expected. However, in the endometrial cancer model, apple seeds induced apoptosis specifically in the endometrium and myometrium. It also enhanced the immune system by upregulating IL-2 expression. In our study, we could not identify the active component responsible for the observed effects. However, we were able to get hints from a study of Annona squamosa L, known as the tropical apple. In other words, the results of this study, as in Chen et al. (2016) study, suggested that the potential effect of alleviating antioxidant and cytotoxic activity in seed-rich vegetable oils is expected to play a role in standard cell populations and negatively affect cancer cells.

### *Conclusion*

This study analyzed whether the administration of apple seeds in t a mouse model of endometrial cancer metastasized from colorectal cancer could suppress endometrial cancer. These results confirmed that the administration of apple seed increased the mechanism of TNFα/p53 pathway-related in the endometrial cancer mouse model and was also involved in morphological changes of endometrial. In addition, results suggest that apple seeds have a differential effect in normal tissue and endometrial cancer tissue and may regulate the

induction of apoptosis. In conclusion, the administration of apple may have the ability to facilitate the reconstruction of normal tissue in endometrial cancer that has metastasized from colorectal cancer.

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