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ORIGINAL ARTICLE

Grape seed proanthocyanidin extract induces apoptotic and autophagic cell death in rheumatoid arthritis fibroblast-like synoviocytes

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ABSTRACT

Objectives: In this study, we aimed to evaluate the association between grape seed proanthocyanidin extract (GSPE) and rheumatoid arthritis-fibroblast-like synoviocytes (RA-FLSs) and to investigate whether GSPE induces cell death in RA-FLSs.

Materials and methods: The FLSs were isolated from RA synovial tissues. Cell viability and cell cycle staging were analyzed using a hemocytometer and flow cytometry. Caspase 3 and poly (ADP-ribose) polymerase (PARP) proteins were analyzed using Western blotting with z-VAD-fmk. Protein LC3 and polyubiquitin-binding protein p62 that were degraded by autophagy were evaluated using Western blotting with 3-methyladenine and chloroquine. Reactive oxygen species (ROS) were also evaluated.

Results: When RA-FLSs were treated with GSPE, cell viability decreased, the number of cells in sub-G1 and G2/M phases increased, and the expression of pro-PARP and pro-caspase 3 proteins decreased in a concentration-dependent manner. This result was offset, when the cells were co-treated with the pan-caspase inhibitor z-VAD-fmk. The reduced cell viability, increased expression of LC3-II protein, and reduced expression of p62 protein with GSPE treatment were offset, when RA-FLSs were co-treated with GSPE and autophagy inhibitors 3-methyladenine and chloroquine. The level of ROS in RA-FLSs treated with GSPE was significantly lower than treatment with N-acetyl-cysteine, a ROS inhibitor.

Conclusion: Our study results show that GSPE induces apoptotic and autophagic cell death and inhibites reactive oxygen species in RA-FLSs.

Keywords: Apoptosis; autophagy, fibroblast-like synoviocytes, grape seed proanthocyanidin extract, rheumatoid arthritis.

Rheumatoid arthritis (RA) is a chronic inflammatory systemic disease characterized by bone and cartilage destruction that can be attributed to continuous synovial inflammatory responses.[1] T cells and B cells play an important role in the pathogenesis of RA. Patients with RA produce about three to four times more autoreactive naive B cells than non-patients. Autoreactive B cells activate T cells by the rheumatoid factor, anti-citrullinated protein antibodies (ACPA) production, pro-inflammatory cytokine production (e.g., tumor necrosis factor-alpha [TNF-α], interleukin [IL]-6, and IL-1α), and costimulatory molecules.[2] Activated T cells produce inflammatory cytokines and destroy synovial tissue.[3] In particular, Th1 secretes IFN-γ, IL-2, and TNF-α and activates macrophages. The Th17 secretes IL-17, which stimulates the production of inflammatory cytokines, chemokines, and matrix metalloproteinase (MMPs).[4] The IL-17 contributes

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to the formation of pannus tissue, as well as the creation of new blood vessels and osteoclasts by overexpression of synovial cells, and it improves the production of IL-6, MMP-1, and MMP-3. This activated immune response results in an abnormal modification of the synovial environment. Fibroblast-like synoviocytes (FLSs) are the part of the structure of the synovial intima and play an important role in the onset of RA.[1] Unlike normal FLSs, the environment of RA-FLSs is changed to high-pressure and low-oxygen conditions, which results in aggressive phenotypes and abnormal proliferation of FLSs.[5] This characteristic results from resistance to apoptosis[6] and is influenced by the intricate interactions of the overproduction of reactive oxygen species (ROS) and the survival mechanism of autophagy.[7,8] Therefore, treatments targeting the characteristics of RA-FLSs are likely to inhibit RA effectively.[1]

Grape seed proanthocyanidin extract (GSPE) consists of polyphenolic compounds with various biological functions. Proanthocyanidin is known to have little toxicity and has powerful antioxidant properties.[9] Previous studies have shown that GSPE suppresses angiogenesis and controls the cell cycle, as well as the lifespan of cells including apoptosis and autophagy in various cancer cell lines such as colorectal, cervical, nasopharyngeal, pancreatic, and stomach cancer.[10-16] Also, the GSPE is known to reduce the severity of RA-FLSs and collagen-induced arthritis by reducing inflammatory cytokines and inhibiting differentiation of osteoclasts.[17-20] However, no studies have focused on the effects of GSPE on cell death pathways in RA-FLSs.

The RA-FLSs show aggressive phenotypes, abnormal proliferation, and microenvironments that are similar to cancer cells.[5] Based on the characteristics of FLSs and the findings of existing anti-cancer and anti-rheumatism studies, we hypothesized that GSPE suppresses the multiplication of RA-FLSs. In the literature, there are no studies on cell death with a focus on the association between GSPE and RA-FLSs. In the present study, we, therefore, aimed to investigate the effects of GSPE and mechanisms of its inhibition of cell multiplication and cell death in RA-FLSs that have similar characteristics to cancer cells.

MATERIALS AND METHODS

Patients

This *in-vitro* study was conducted at Keimyunng University, Department of Rheumatology between January 2020 and December 2020.

The samples of RA synovial tissues were obtained through synovectomy from RA patients who met the diagnostic criteria established by the American College of Rheumatology in 1987.[21] To evaluate RA, erythrocyte sedimentation rate, C-reactive protein, rheumatoid factor, and anti-cyclic citrullinated peptide antibody were measured.

RA-FLS isolation

To isolate cells from the collected synovial tissues, fat was removed from the tissues and the remaining tissues were finely cut with alcohol-sterilized scissors. These pieces were, then, cultured at 37°C for 2 h in serum-free Dulbecco's minimum Eagle medium (DMEM; Welgene Inc., Gyeongsan-si, Republic of Korea) supplemented with collagenase (0.5 mg/mL; Gibco, Waltham, MA, USA). To obtain the cell suspension only, the pieces were filtered through sterilized gauze and, then, centrifuged at 4,500×g for 5 min. The deposit was centrifuged at 4,500×g for 5 min after being washed in phosphate-buffered saline (PBS). This process was repeated twice. The deposit was cultured in DMEM supplemented with 1% penicillin-streptomycin (HyClone, Logan, UT, USA) and 10% fetal bovine serum (Welgene Inc., Gyeongsan-si, Republic of Korea) at 37°C in a 5% CO_2 incubator.[22] In addition, FLSs that were subcultured four to seven times were used to avoid contamination of lymphocytes and macrophages.[23]

Reagents

The GSPE was provided by Hanlim Pharm (Seoul, Republic of Korea). Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk) was purchased from R&D Systems (Minneapolis, MN, USA); 3-methyladenine (3MA), chloroquine diphosphate salt solid (CQ), N-acetyl-cysteine (NAC), 2',7'-dichlorofluorescein diacetate (DCFH-DA), and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). In addition, lysis buffers were purchased from Thermo Fisher (Waltham, MA, USA),

and protease and phosphatase inhibitors were purchased from Roche Diagnostics GmbH (Mannheim, Germany).

Cell viability assay

To evaluate cell viability, the RA-FLSs were seeded on six-well plates at a density of 2×10^5 cells/mL and, then, treated with GSPE. After the collected cells were dyed with 0.2% trypan blue, the number of undyed cells were counted using a hemocytometer and a light microscope (EVOS™ XL Core Imaging System, Thermo Fisher Scientific, Waltham, MA, USA). The RA-FLSs were seeded three times, and the number of cells were counted three times for each independent experiment.

Cell cycle analysis

After RA-FLSs were seeded on 60-mm plates at a density of 4×10^5 cells/mL and treated with GSPE and z-VAD-fmk, they were cultured at 37°C in a 5% CO_2 incubator for 16 h. The cultured cells were obtained and fixed with cold 70% ethanol at 4°C for 24 h. After the fixed cells were washed with PBS, their supernatants were removed after centrifugation at 4,500×g for 3 min. The cells were treated with a PI solution (2.5 mg/mL PI, 5 mg/mL RNase A, 0.1% NP-40, 0.1% trisodium citrate) and allowed to react for 20 min at 4°C, while light was blocked. The cycles of the resulting cells were analyzed using the flow cytometer BD FACSCanto II (BD Biosciences, Franklin Lakes, NJ, USA). Each independent experiment was conducted three times.

Western blot

The RA-FLSs were seeded on six-well plates at a density of 2×10^5 cells/mL and, then, treated with GSPE. After the cells were washed with PBS, lysis buffers as well as protease and phosphatase inhibitors were added and the cells were kept on ice and allowed to react for 15 min and, then, moved to new tubes. To obtain protein supernatants, the cells were centrifuged at 4°C and 16,000×g for 15 min. Equal amounts of protein (30 μg) were aliquoted following protein quantitation by the bicinchoninic acid assay (Thermo Fisher Scientific, Rockford, IL, USA). Samples were mixed with a sodium dodecyl sulfate (SDS) loading buffer and separated with PageRuler Plus Prestained Protein Ladder (Thermo Fisher Scientific) using SDS polyacrylamide gel electrophoresis (Western Blotting Kit, Hoefer Inc., MA, USA). Proteins were, then, transferred onto nitrocellulose membranes (Amersham, Chicago, IL, USA). The membranes were blocked at room temperature for at least 1 h using Tris-buffered saline with 0.1% Tween 20 supplemented with 5% skim milk (20 mM Tris, 137 mM NaCl, 0.05% Tween 20) and, then, allowed to react with the primary antibodies overnight at 4°C. After washing the membranes with primary antibodies using TBS-T three times in 10-min intervals, they were reacted with secondary antibodies at room temperature for 1 h. After washing the reacted membranes, they were illuminated with an Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA) solution to identify the expression of specific proteins with a chemiluminescence image analyzer (Fusion Solo 6S, Vilber Lourmat, Marne-la-Vallée, France).

Regarding primary antibodies, β-actin (1:5,000) and p62/SQSTM1 (1:8,000) antibodies were purchased from Sigma-Aldrich. The LC3 (1:2000) and pro-caspase 3 (1:2,000) antibodies were supplied from MBL International (Woburn, MA, USA) and Santa Cruz Biotechnology (Dallas, TX, USA), respectively. In addition, poly (ADP-ribose) polymerase (PARP; 1:1,000) and cleaved-caspase 3 (1:1,000) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). For secondary antibodies, Peroxidase AffiniPure Donkey Anti-Mouse IgG (H+L; 1:5,000) and Peroxidase-Conjugated AffiniPure Donkey Anti-Rabbit IgG (H+L; 1:2,000) antibodies were purchased from Jackson ImmunoResearch (West Grove, PA, USA). The U87MG cells, a glioblastoma cell line treated with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) that selectively induces apoptosis in cancer cells, were used as a loading control to confirm the expression of apoptosis-related proteins. Each independent Western blot was conducted three times.

Analysis of ROS levels

After RA-FLSs were seeded on 60-mm plates at a density of 4×10^5 cells/mL, they were treated

with GSPE and NAC and, then, cultured for 16 h at 37°C in a 5% CO_2 incubator. The cells were obtained and treated with DCFH-DA (30 μM) and, then, allowed to react for 30 min at 37°C and 5% CO_2, while light was blocked. After the resulting cells were washed with PBS and the supernatants from the cells were removed after centrifugation at 4,500×g for 3 min. After the cells were suspended in PBS, changes in the amount of ROS were analyzed using the flow cytometer BD FACSCanto II (BD Biosciences, Franklin Lakes, NJ, USA). Each independent experiment was conducted three times.

Statistical analysis

Statistical analysis was performed using the IBM SPSS version 25.0 software (IBM Corp., Armonk, NY, USA). Descriptive data were expressed in mean ± standard deviation (SD). The significance of the results was analyzed using one-way analysis of variance (ANOVA), followed by Bonferroni post-hoc comparisons test. A p value of <0.05 was considered statistically significant.

RESULTS

Decrease in cell viability caused by GSPE

Changes in the number of surviving cells were examined to identify whether GSPE could suppress the multiplication of RA-FLSs. Examination of cell viability after treatment with GSPE at concentrations of 100 to 400 μg/mL revealed that cell viability reduced with the increased GSPE concentration (Figure 1a, f=13.53, p<0.001). When treated with GSPE at 200 μg/mL for 4 to 24 h, RA-FLSs showed a statistically significant decrease in their viability at 16 h (Figure 1b, f=63.21, p<0.001). When treated with GSPE at 50 to 200 μg/mL for 16 h, the RA-FLSs exhibited statistically significant decreases in the survival rates at 17% (50 μg/mL), 34% (100 μg/mL), and 53% (200 μg/mL) increases in GSPE concentration (Figure 1c, f=67.54, p<0.001). These results confirmed that GSPE treatment had an inhibitory effect on the multiplication of RA-FLSs.

Apoptosis-inducing effects of GSPE

We investigated whether the suppression of the survival of RA-FLSs by GSPE was due to its apoptosis-inducing effects. When changes in cell viability were examined after RA-FLSs were pre-treated with z-VAD-fmk (50 μM), a pan-caspase inhibitor, for 30 min and treated with GSPE (200 μg/mL) for 16 h, cell viability, which decreased with GSPE treatment alone, was significantly restored with z-VAD-fmk co-treatment (Figure 2a, f=44.27, p<0.001).

The cell cycle stage was determined using a flow cytometer. When RA-FLSs were treated with GSPE at 50 to 200 μg/mL for 16 h, the number of cells in sub-G1 phase increased to 7.2%, 11.5%, and 13.1% depending on the concentration (50, 100, or 200 μg/mL) of GSPE,

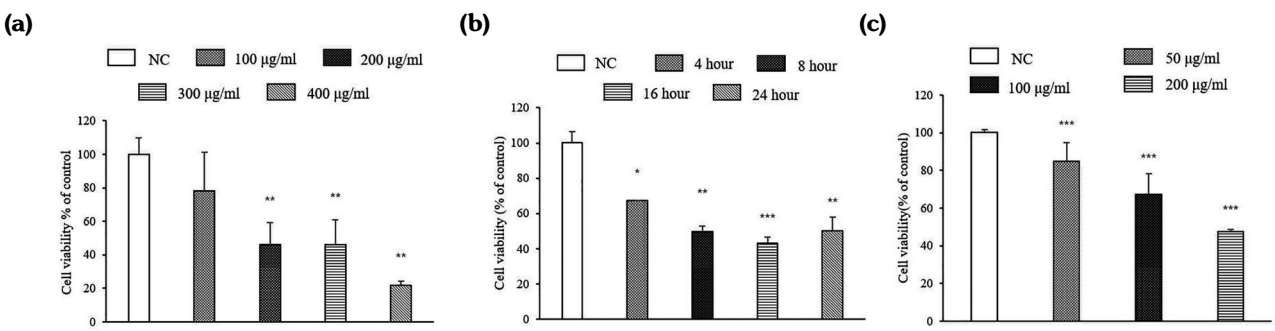

Figure 1. GSPE reduces cell viability in RA-FLSs. **(a)** RA-FLSs were treated with GSPE at various concentrations (0 to 400 μg/mL) for 24 h. **(b)** The cell viability of GSPE-treated (200 μg/mL). RA-FLSs was evaluated at various time points (4 to 24 h). **(c)** RA-FLSs were treated with GSPE at various concentrations (0 to 200 μg/mL) for 16 h. Cell viability was determined with a hemocytometer using trypan blue staining.

Error bars indicate mean ± standard deviation (SD). * p<0.05; ** p<0.01; *** p<0.001, compared to control cells. GSPE: Grape seed proanthocyanidin extract; RA-FLSs: Rheumatoid arthritis fibroblast-like synoviocytes.

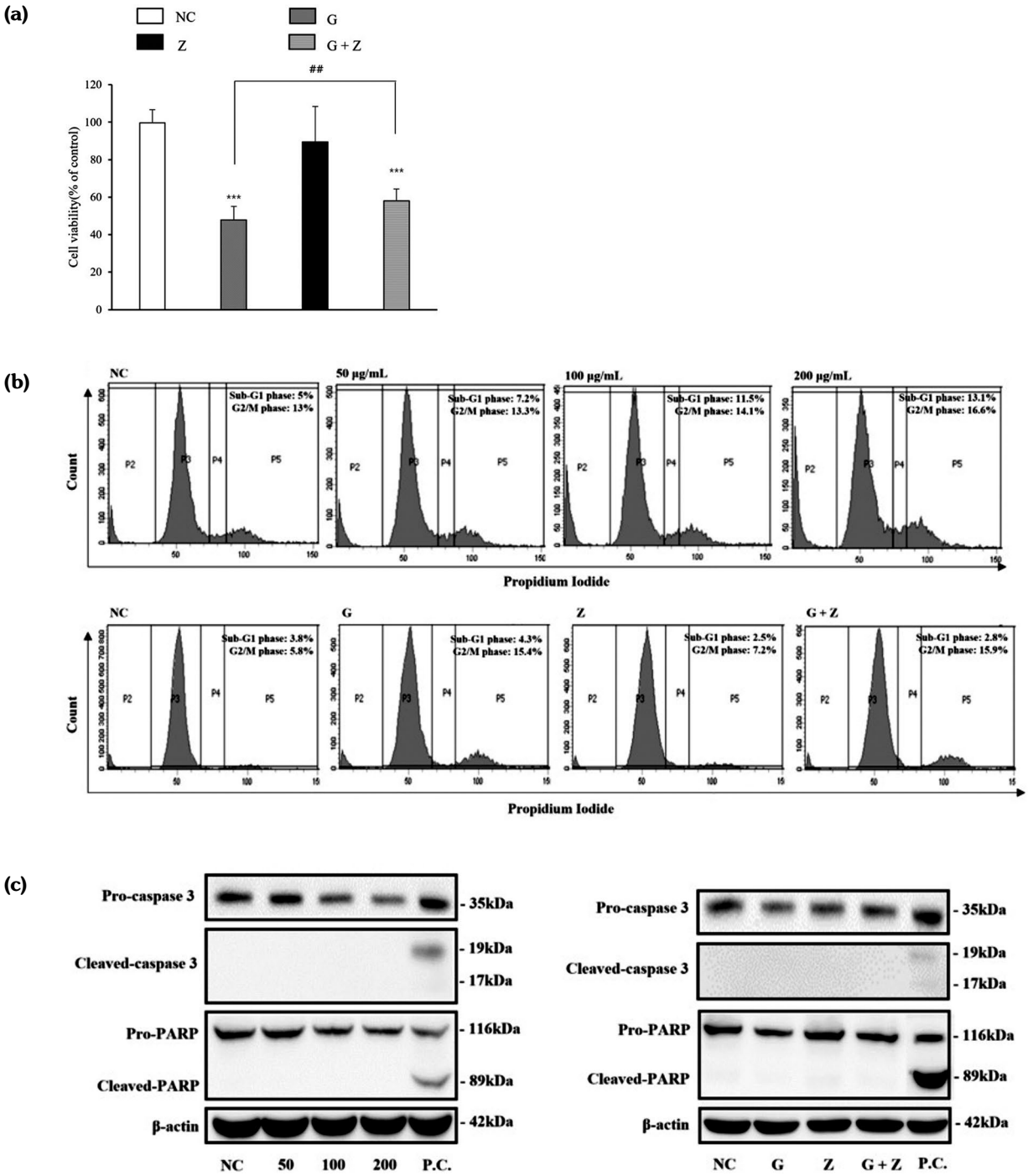

Figure 2. GSPE induces apoptosis in RA-FLSs. **(a)** RA-FLSs were pre-treated with the pan-caspase inhibitor z-VAD-fmk (50 μM) for 30 min before the addition of GSPE (200 μg/mL) for 16 h. Cell viability was determined with a hemocytometer using trypan blue staining. **(b)** RA-FLSs were treated with GSPE (50-200 μg/mL) and pre-treated with or without z-VAD-fmk (50 μM) for 30 min and, then, incubated with GSPE (200 μg/mL) for 16 h. RA-FLSs were stained with propidium iodide and their cell cycle stage was determined by flow cytometry. **(c)** Western blot analysis was performed using PARP, pro-caspase 3, cleaved-caspase 3 antibodies. β-actin was used as a loading control. U87MG cells treated with tumor necrosis factor-related apoptosis-inducing ligand were used as a PC.

GSPE: Grape seed proanthocyanidin extract; RA-FLSs: Rheumatoid arthritis fibroblast-like synoviocytes; z-VAD-fmk: Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; PARP: Poly (ADP-ribose) polymerase; PC: Positive control; Error bars indicate mean ± standard deviation (SD); *** $p<0.001$, compared to control cells; ## $p<0.01$, compared to GSPE-treated cells.

respectively (Figure 2b). The number of cells in G2/M phase also increased to 13.3%, 14.1%, and 16.6%, respectively. According to the results of flow cytometry of co-treatment of cells with GSPE and z-VAD-fmk under the same conditions, the number of cells in sub-G1 phase, which increased with GSPE treatment alone, decreased with z-VAD-fmk co-treatment (Figure 2b). In addition, the expression of major apoptosis proteins PARP (89, 116 kDa), pro-caspase 3 (35 kDa), and cleaved-caspase 3 (17, 19 kDa) were examined using Western blotting. When RA-FLSs were treated with GSPE at 50 to 200 µg/mL, the expression of pro-PARP and pro-caspase 3 decreased in a concentration-dependent manner, but the expression of cleaved-caspase 3 and cleaved-PARP was not detected. When RA-FLSs were co-treated with z-VAD-fmk (50 µM) and GSPE, the expression of pro-PARP, which decreased with GSPE treatment alone, increased after z-VAD-fmk co-treatment. U87MG cells treated with TRAIL detected cleaved-PARP and cleaved-caspase 3 expression, but the same was not detected in RA-FLSs (Figure 2c). These results showed that GSPE was involved in the induction of apoptosis accompanied by a G2/M phase arrest in RA-FLSs.

Autophagy-inducing effects of GSPE

In this study, we investigated whether autophagy was involved in the inhibitory effect of GSPE on cell proliferation in RA-FLSs. After RA-FLSs were treated with GSPE at 50 to 200 µg/mL for 16 h, the expression of autophagy marker proteins LC3 (16, 18 kDa) and p62 (62 kDa) was examined using Western blotting. An increase in the concentration of GSPE resulted in a corresponding increase in the expression of LC3-II and a corresponding

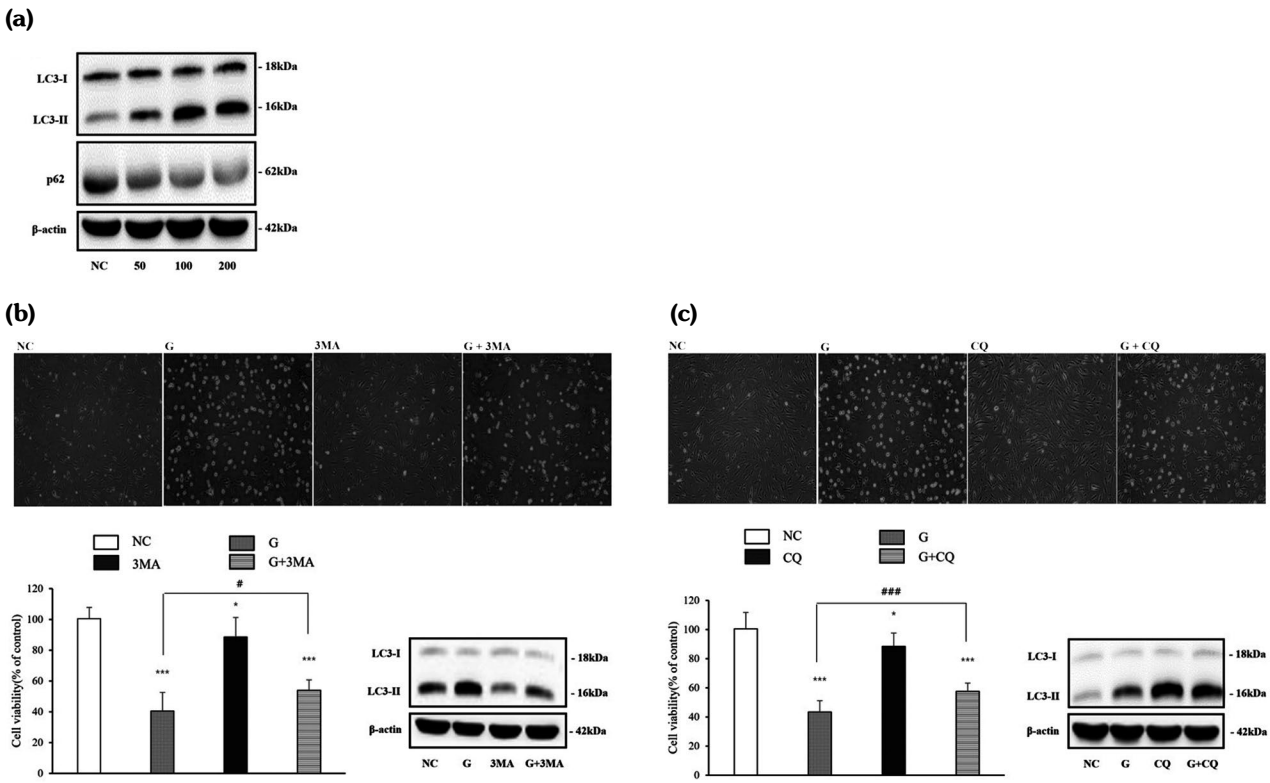

Figure 3. GSPE induces autophagy in RA-FLSs. **(a)** RA-FLSs were treated with GSPE at various concentrations (0 to 200 µg/mL) for 16 h. Western blot analysis was performed using anti-LC3 and anti-p62 antibodies. β-actin was used as a loading control. **(b, c)** RA-FLSs were co-treated with GSPE (200 µg/mL) in the presence or absence of autophagy inhibitors (500 µM 3MA or 20 µM CQ) for 16 h. Cell morphology was photographed using a light microscope (×200). Cell viability was determined with a hemocytometer using trypan blue staining.

Error bars indicate means ± standard deviation (SD); * $p<0.05$ and *** $p<0.001$, compared with control cells. # $p<0.05$ and ### $p<0.001$, compared to GSPE-treated cells. Western blot analysis was performed using anti-LC3 antibody. β-actin was used as a loading control. GSPE: Grape seed proanthocyanidin extract; RA-FLSs: Rheumatoid arthritis fibroblast-like synoviocytes; 3MA: 3-methyladenine; CQ: Chloroquine diphosphate salt solid.

decrease in the expression of p62 (Figure 3a). In addition, RA-FLSs were treated with 3MA, which inhibits phosphatidylinositol 3-kinase (PI3K), a protein that activates autophagy, and CQ, which inhibits the binding of autophagosomes to lysosomes. After treatment, cellular changes were observed with a light microscope and, then, the number of surviving cells were counted, and the expression of LC3-II was examined. The RA-FLSs were treated with GSPE (200 μg/mL) alone or in combination with 3MA (500 μM), which is an early-stage inhibitor of autophagy, for 16 h. Increased dead cell suspensions, reduced cell viability, and increased expression of LC3-II were found with GSPE treatment alone; however, decreased dead cell suspensions, restored cell viability, and reduced expression of LC3-II were found with the 3MA co-treatment (Figure 3b, f=71.00, p<0.001). Similarly, RA-FLSs were treated with GSPE (200 μg/mL) alone or in combination with CQ (20 μM), a late-state inhibitor for autophagy, for 16 h. Increased dead cell suspensions, reduced cell viability, and the accumulated expression of LC3-II were observed with GSPE treatment alone, whereas decreased dead cell suspensions, restored cell viability, and further accumulated expression of LC3-II were detected with CQ co-treatment (Figure 3C, f=83.50, p<0.001). These results confirmed that GSPE

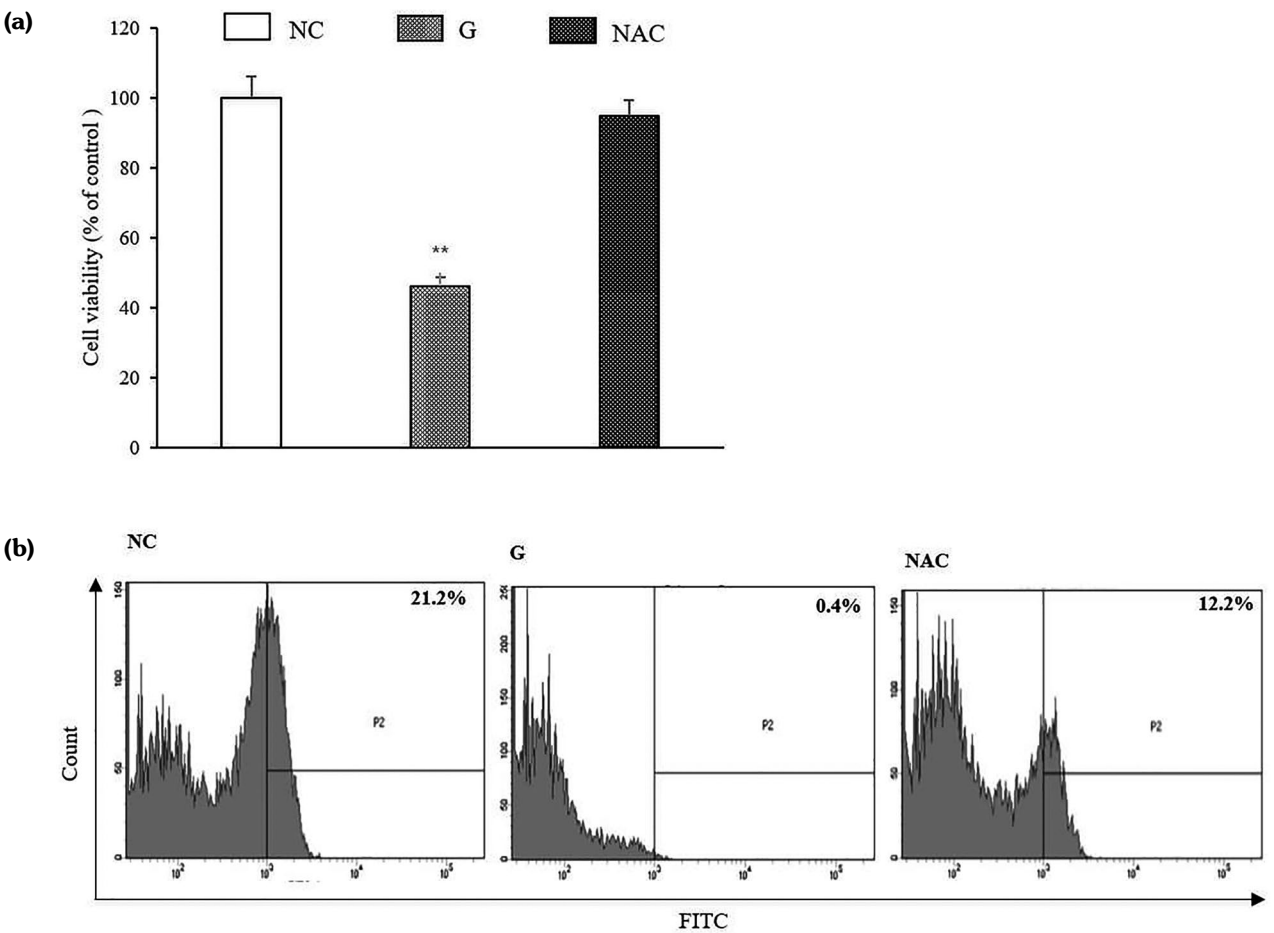

Figure 4. GSPE scavenges reactive oxygen species in RA-FLSs. **(a)** RA-FLSs were treated with GSPE (200 μg/mL) and NAC (10 mM) for 16 h. Cell viability was determined with a hemocytometer using trypan blue staining. **(b)** RA-FLS were treated with GSPE (100 μg/mL) and NAC (10 mM) for 8 h. The intracellular reactive oxygen species level was analyzed by flow cytometry using DCFH-DA (30 μM).

GSPE: Grape seed proanthocyanidin extract; RA-FLSs: Rheumatoid arthritis fibroblast-like synoviocytes; NAC: N-acetyl-cysteine; FITC: Fluorescein isothiocyanate; DCFH-DA: 2',7'-dichlorofluorescein diacetate.

was involved in the induction of cell death in RA-FLSs through autophagy.

Effects of GSPE on ROS

To determine the antioxidant effects of GSPE on RA-FLSs, the ROS inhibitor NAC was used for the positive control group to observe changes in the number of surviving cells. As a result of co-treating RA-FLSs with GSPE (200 μg/mL) and NAC (10 mM), the cells showed reduced cell viability when treated with GSPE, but exhibited no changes in cell viability when treated with NAC compared to the control group (Figure 4a, $f=122.20$, $p<0.001$). This result indicated that NAC did not show cytotoxicity in RA-FLSs. When RA-FLSs were treated with GSPE (100 μg/mL) and NAC (10 mM) for 8 h and analyzed using flow cytometry to examine changes in the amount of ROS, we found that ROS reduced 1.7-fold more than the control group in the case of NAC treatment and 53 times more than the control group in case of GSPE treatment (Figure 4b). This outcome suggested that GSPE reduced the generation of ROS in RA-FLSs to a greater extent than NAC.

DISCUSSION

Rheumatoid arthritis develops from bone and cartilage destruction caused by abnormal proliferation of synovia.[1] The RA-FLSs are characterized by the overproduction of inflammatory cytokines, high-pressure and hypoxic microenvironments,[5] and show resistance to apoptosis.[6] A known mechanism underlying the resistance of RA-FLSs to apoptosis involves mutation of p53, microenvironments that are rich in active nitrogen and oxygen, and the activation of NF-κB resulting from the relatively low expression of phosphatase and a tensin homolog that induce a decrease in apoptosis.[1] These characteristics can be stimulated by the production of ROS and the resulting modified biological imbalance can induce the survival mechanism of autophagy.[11]

Cell death pathways in the form of apoptosis and autophagy play a vital role in maintaining cellular homeostasis.[24] Occasionally, apoptosis induces the death of cells in cooperation with autophagy, and autophagy suppresses apoptosis by facilitating the survival of cells.[25] Apoptosis can be initiated by one of two pathways: the extrinsic pathway related to the activation of cell death receptors and the intrinsic pathway via mitochondria. These two pathways are connected through caspase 3, thereby inducing cell death.[26] Autophagy is a mechanism that breaks down unnecessary cell organelles or proteins through the fusion of autophagosomes and lysosomes.[27] Although it works as a mechanism that protects cells under stress, it can cause cell death when this defense mechanism does not overcome excessive stress.[28]

The GSPE is a powerful antioxidant with proanthocyanidin complexes and is known to have a cytoprotective effect in oxidative stress-borne metabolic disorders.[29,30] According to previous studies, GSPE induces an inhibitory effect on arthritis through the Toll-like receptor-4 signal transduction pathway,[17] and suppresses inflammatory cytokines and bone destruction caused by the imbalance of osteoclasts.[20] The GSPE increases glucocorticoid-induced tumor necrosis factor receptor (GITR) cell expression, a subset of T cells, in an animal model of RA.[31] It also increases the number of Th2 cells as well as IL-4 and IL-10 produced by Th2 cells.[19] However, it can decrease the number of Th1/Th17 cells as well as IL-2, IFN-γ, IL-6, and TNF-α produced by Th1/Th17.[32] In addition, CD4+, CD25+, and Treg play an important role in regulating and preventing autoimmunity and in preventing tissue damage by inhibiting proliferation of Th1/Th17 cells. The GSPE is known to inhibit cell multiplication by inducing apoptosis and autophagy simultaneously in some cancer cells.[12,33]

In this study, GSPE significantly inhibited the survival of RA-FLSs. The suppression of cell survival was restored, when the cells were co-treated with the pan-caspase inhibitor z-VAD-fmk and GSPE. When the cell cycle stage was determined after RA-FLSs were treated with GSPE, the number of cells in sub-G1 and G2/M phases increased depending on the concentration of GSPE and the increased number of cells in sub-G1 phase were decreased by the co-treatment with z-VAD-fmk. The expression of apoptosis markers pro-PARP and pro-caspase 3 decreased depending on the concentration of GSPE, and the decreased expression of pro-PARP increased with co-treatment with z-VAD-fmk. These findings demonstrate that GSPE induces apoptosis in

RA-FLSs, but the partial expression of apoptosis protein markers is likely to induce apoptosis in association with other mechanisms of cell death.

When the expression of protein autophagy markers was examined after RA-FLSs were treated with GSPE, increases in the expression of LC3-II and decreases in the expression of p62 were detected. The increased expression of LC3-II indicates normal formation of autophagosomes. p62 is an adapter protein that is degraded, when ubiquitinated protein aggregates are degraded via LC3 to remove damaged organelles.[34] In this study, when GSPE was treated with RA-FLS, the expression of LC3-II protein was increased in a concentration-dependent manner, resulting in induction of the initial stage of autophagy. In addition, the late stage of autophagy was induced by GSPE, while the expression of p62 protein was decreased. To understand how autophagy was induced, RA-FLSs were co-treated with 3MA, which inhibits the early stages of autophagy and CQ, which inhibits the late stages of autophagy, and changes in cell viability and protein expression were observed. As a result, the decreased cell viability with GSPE treatment alone was restored through co-treatment with autophagy inhibitors and GSPE, and the increased expression of LC3-II was reduced by 3MA and increased by CQ. 3MA blocks the formation of autophagosomes by suppressing PI3K, thereby suppressing LC3-II, and CQ inhibits the fusion of autophagosomes and lysosomes, which results in the accumulated expression of LC3-II.[35] Given these results, we concluded that GSPE induced cell death in RA-FLS through autophagy.

Regarding the role of ROS in the process of cell death through GSPE, conflicting results have been reported according to cell type.[36-38] Rheumatoid arthritis causes the accumulation of ROS due to an imbalance in oxidative stress and, thus, affects the onset of inflammation and the multiplication of synovia.[6] A study reported that resveratrol, which is a polyphenol antioxidant similar to GSPE, suppressed the production of ROS in RA-FLSs, thereby inducing apoptosis and inhibiting cell multiplication.[39] In the present study, changes in the cell viability and ROS were observed using a control group with the ROS inhibitor NAC, based on the assumption that GSPE would induce cell death through its antioxidant activity. As a result, cell viability was reduced with GSPE treatment, but no changes were found with the NAC treatment. In addition, a significant decrease in ROS was detected with GSPE treatment compared to NAC treatment. These findings indicate that GSPE influences ROS reduction in RA-FLSs, but it would still be necessary to compare the significance of antioxidant effects of GSPE using antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxide, other than NAC, and some natural antioxidants.

In conclusion, our study results demonstrate that GSPE suppresses cell survival through apoptosis and autophagy in RA-FLSs and induces a reduction in ROS and, thus, may play a significant role in RA treatment.

Ethics Committee Approval: The study protocol was approved by the Institutional Review Board of Keimyung University Dongsan Hospital (No: 2020-01-055). The study was conducted in accordance with the principles of the Declaration of Helsinki.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions: All authors contributed equally to the article.

Conflict of Interest: The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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