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ARAŞTIRMA / RESEARCH

Ganoderma lucidum inhibits proliferation and promotes autophagy in breast cancer cell lines with varying hormonal sensitivity by regulating Beclin-1, LC3, and p62

Ganoderma lucidum farklı hormonal duyarlılığa sahip meme kanseri hücre hatlarında Beclin-1/LC3 ve p62'yi düzenleyerek proliferasyonu inhibe eder ve otofajiyi teşvik eder

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Abstract

Purpose: The aim of this study was to investigate the role of Ganoderma lucidum (GL) in cell proliferation and autophagy in breast cancer cell lines.
Materials and Methods: Cell lines were cultured in vitro and the appropriate dose was determined by the MTT method. In order to show the effect of GL on autophagy in MCF-7 and MDA-MB-231 breast cancer cell lines, Beclin-1, LC3 and p62 expression were examined by immunohistochemistry staining. Additionally, cell cycle analysis was carried out in GL-treated and untreated cells using a cell cycle analysis kit to look into how GL affects cell division.
Results: Viability level was significantly reduced in MCF-7 and MDA-MB-231 cells incubated with 40 and 80 µM concentrations of GL for 24 hours. In addition, GL induced the initiation of autophagy in MDA-MB-231 and MCF-7 cells, as evidenced by enhanced levels of Beclin-1, LC3 and p62 expression. At the same time, this study showed that GL induced cell cycle arrest in the G2/M phases.
Conclusion: GL has anti-proliferative activity by arresting the cell cycle at the G2/M phase and accelerates cell death by inducing autophagy, particularly in aggressive breast cancers that are resistant to treatment..
Keywords: Ganoderma lucidum, MCF-7, MDA-MB-231, autophagy, cell cycle

Öz

Amaç: Bu çalışmada meme kanseri hücre dizilerinde Ganoderma lucidumun (GL) hücre proliferasyonu ve otofaji ile ilişkisinin belirlenmesi amaçlanmıştır.
Gereç ve Yöntem: Hücre hatları in vitro ortamda kültüre edildi ve uygun doz MTT yöntemi ile belirlendi. MCF-7 ve MDA-MB-231 meme kanseri hücre hatlarında GL'nin otofaji üzerindeki etkisini göstermek için, immünohistokimya boyaması ile Beclin-1, LC3 ve p62 ekspresyonu incelenmiştir. Ek olarak, GL'nin hücre bölünmesi üzerindeki etkisini araştırmak için, bir hücre döngüsü analiz kiti kullanılarak GL uygulanan ve uygulanmayan hücre döngüsü analizi yapıldı.
Bulgular: 24 saat süreyle GL'nin 40 ve 80 µM'lik konsantrasyonlarıyla inkübe edilen MCF-7 ve MDA-MB-231 hücrelerinde canlılık düzeyinin önemli ölçüde azaldığı gösterildi. Ek olarak, GL, artan Beclin-1, LC3 ve p62 ekspresyonu seviyeleri ile kanıtlandığı gibi, MCF-7 ve MDA-MB-231 hücrelerinde otofajinin başlatılmasını indüklemiştir. Aynı zamanda, bu çalışma GL'nin G2/M fazlarında hücre döngüsü durmasını indüklediğini gösterdi.
Sonuç: GL, hücre döngüsünü G2/M fazında durdurarak anti-proliferatif aktiviteye sahiptir ve özellikle tedaviye dirençli agresif meme kanserlerinde otofajiyi indükleyerek hücre ölümünü hızlandırır.
Anahtar kelimeler: Ganoderma lucidum, MCF-7, MDA-MB-231, otofaji, hücre döngüsü

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INTRODUCTION

Breast cancer is a heterogeneous disease with many subtypes that have different histopathological models and molecular properties, resulting in a variety of therapy options and clinical charts[1]. Traditionally, treatment of breast cancer is planned according to the expression of hormone receptors such as estrogen, progesterone and human epidermal growth factor. The presence of these receptors has allowed the development of targeted and effective therapies[2]. MCF-7 is an estrogen receptor-positive (ER+) breast cancer subtype that can be treated with estrogen receptor-inhibiting drugs[3,4]. However, the triple-negative breast cancer cell line MDA-MB-231 expresses neither estrogen, progesterone, nor human epidermal growth factor receptors (HER2)[5]. As a result, this type of breast cancer does not respond to specific treatments that target these receptors, resulting in a lower overall patient survival rate[6]. Therefore, the development of new treatments is exciting and promising.

Ganoderma lucidum (GL), also known as the mushroom of immortality, has many medicinal benefits and has been used for many illnesses in the East Asian region for many years[7], including cancer, immunological disorders, inflammation, and cardiovascular disease. Clinical studies have verified that supplementing chemotherapy, radiotherapy, and surgery with GL can rehabilitate patients' quality of life and decrease side effects caused by chemotherapy and radiotherapy[8]. Also, GL has an anti-cancer action due to the triterpenoids, polysaccharides, and ganoderic acids that it contains, and is known to cause DNA damage effects on cancer cells by triggering apoptosis[9].

The increase in cell number caused by cell division is attempted to be balanced by programmed cell death. Disruption of the balance between cell growth and cell death can lead to malignant proliferation[10, 11]. The primary reason for the emergence of cancer is the disruption of cell cycle mechanisms and the continuous proliferation of cancer cells[12,13]. Autophagy, also known as type 2 cell death, is a catabolic process that digests self-components. Its literal meaning is "self-eating." This degradation system is essential to maintaining cellular homeostasis in cases of starvation or damage[14,15]. Autophagy is closely linked to many diseases, such as cardiovascular disease, neurodegenerative disorders, obesity, aging, and cancer[16, 17]. The role of autophagy in cancer is complicated and unclear. Its mechanism may differ depending on the type of cancer. For example, in some cancer types, it may inhibit the initiation and progression of the tumor, while in others it may promote tumor survival and progression. Given these circumstances, investigation of autophagy in cancer is promising but challenging[18]. Our study examined the effect of varying GL concentrations on the proliferation of different breast cancer cell lines. To investigate the mode of cell death via autophagy, we selected MCF-7 and MDA-MB-231 human breast cancer cells. The aim of this study was to evaluate in vitro the effect of GL on the hormonal sensitivity of two different cell lines (MCF-7 and MDA-MB-231), including autophagy induction, cell cycle arrest, and related molecular mechanisms in these cell lines.

MATERIALS AND METHODS

Preparation of GL

The experimental stages of the study took place at the Erciyes University Genome and Stem Cell Center (GENKOK), and ethics committee approval is not required as it is a cell culture study on cell lines.

GL was purchased from Sigma (Sigma Aldrich, 1288372-1G, St. Louis, USA). The stock solution of GL was prepared with 100% sterile dimethylsulfoxide (DMSO, Sigma Aldrich) and stored at +4 °C[19]. It was diluted with FBS-free Dulbecco modified Eagle medium (DMEM; Sigma-Aldrich, St. Louis, MO) according to the determined doses (40, 80, and 100 μM) and applied to cells.

Cell culture

MCF-7 and MDA-MB-231 cell lines were acquired from the American Type Culture Collection (Manassas, VA, USA). The cell lines were cultured in DMEM, which included 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO), 10% penicillin (100 units/ml) and streptomycin (100 μg/ml) and 1% L-glutamine. After incubation (37°C, 5% CO_2), cells were evaluated with an inverted microscope for viability and contamination. When the cells filled 80-90% of the flask, they were removed with trypsin-EDTA and passaged. To determine whether a sufficient number of cells were present for the experiments, cells were counted on the thoma slide by staining with trypan blue[20].

Cell viability and proliferation assays

The cell viability analysis of GL treated MCF-7 and MDA-MB-231 cells was quantified using the MTT cell proliferation assay (Promega, Madison, WI). The MTT assay was performed according to the manufacturer's instructions. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide), which is a tetrazolium salt, is used for cell viability, proliferation, and cytotoxicity. Metabolically active cells convert into a purple-colored formazan product and a quantitative result is obtained by measuring absorbance[21]. The cells were seeded in 96-well plates (1.5×10^3 cells/well) and incubated at 37°C overnight. After incubation, different doses of GL (40 µM, 80 µM and 100 µM) were applied to the cells for 24 hours. MTT reagent was added to the cells after 24 hours, and they were incubated at 37°C for 4 hours [21]. The Elisa Reader was used to measure absorbance at 450 nm and provide the results (Promega Glomax Multi Detection System).

Immunofluorescence staining method

An immunofluorescence staining method was preferred to show Beclin-1, LC3 and p62 expressions in both cell lines. Cells fixed on the coverslip were washed with phosphate buffered saline (PBS) and incubated for 60 minutes by adding 5% goat serum to the coverslip. Then, the cells were kept at +4°C for overnight with Beclin-1 (1:200, Novus Biologicals, Catalog No: NB500-249, Littleton, USA), LC3 (1:250, Cell Signaling Technology, Catalog No: 4108S, Danvers, USA) and p62 (1:200, Novus Biologicals, Catalog No: NBP1-48320, Littleton, USA) antibodies used as autophagy markers.

The next day, after washing with PBS, cells were treated with the secondary antibody (1:200, Jackson ImmunoResearch, Cambridgeshire, UK) for 60 minutes at room temperature. As the last step, the cells were stained with DAPI for one minute, and after washing with PBS again, they were made ready for examination with fluorescence mounting medium (Southern Biotechnologies, Birmingham, USA). Visualized cells were examined under the Olympus BX51 (Tokyo, Japan) fluorescence microscope. To determine the immunoreactivity intensity of each primary antibody, images were obtained from 10 separate microscopic fields (400 X) for each group. The intensity of immunoreactivity was measured using Image J software (Bethesda, USA)[22].

Cell cycle analysis

Depending on the endoplasmic reticulum stress, the synthesis of proteins involved in the cell cycle is suppressed, and accordingly, the dividing cells stop dividing and wait in the G1 phase of the cell cycle. Therefore, to investigate the effect of GL on cell division, cell cycle analysis was performed in GL-treated and untreated cells (control cells) using a cell cycle analysis kit (Luminex, Catalog No: MCH100106, Texas, US) suitable for the muse device. Cells treated with GL were centrifuged at 300×g for 5 min for harvesting from the culture medium and washed with PBS. The cells were then fixed overnight in 70% ethanol at 20 °C. After centrifuging the cells the next day, MuseTM cell cycle reagent was added and they were incubated without light for 30 minutes. Analyses were made with a Muse cell analyzer and Muse analysis software (Merck Millipore)[23].

Statistical analysis

A statistical analysis of the obtained data was performed using the GraphPad Prism 8.0 software program. The conformity of the data to the normal distribution was evaluated with the Shapiro-Wilk test. One sample t test was preferred to compare the percent viability values according to groups. Results were considered significant if the p value was less than 0.05. Each test was run three times (n = 3).

RESULTS

Effect of GL on MCF-7 and MDA-MB-231 cell proliferation

The cytotoxic effects of GL prepared by dissolving in sterile DMSO were evaluated by the MTT analysis method. Different concentrations of GL were applied to the MCF-7 and MDA-MB-231 cells after 24 hours. Cell viability of MCF-7 and MDA-MB-231 cells were found to be significantly reduced at increasing GL concentrations (respectively, $p<0.001$, $p<0.001$). There was a statistically significant decrease in cell viability of 40 µM and 80 µM group at 24 h after GL administration compared to the control group in MCF-7 and MDA-MB-231 cells (respectively, $p<0.001$, $p<0.001$). When the 40 µM group was compared with the 80 µM group, cell viability in the 40 µM group was statistically significantly higher than the 80 µM group in MCF-7 and MDA-MB-231 cells ($p<0.001$).

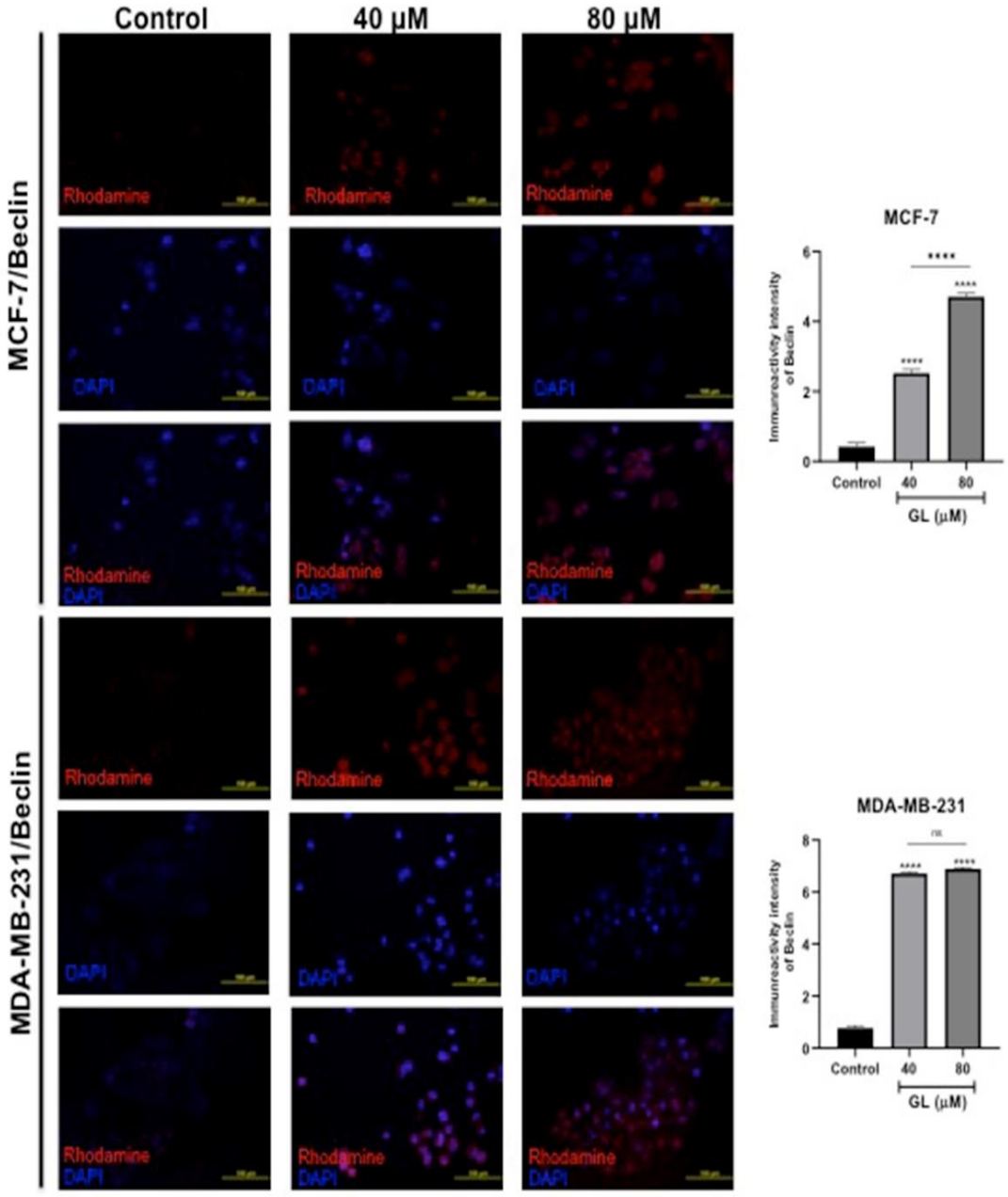

Figure 1. In the MCF-7 and MDA-MB-231 breast cancer cell line treated with GL, Beclin-1 expressions in the groups are seen as red fluorescent reflections, Original magnification, ×400. Graphs show the effect of different doses GL on Beclin-1 protein expression in MCF-7 and MDA-MB-231 breast cancer cells.

(Scale bar: µm, ns: non-significant, ****p<0.0001).

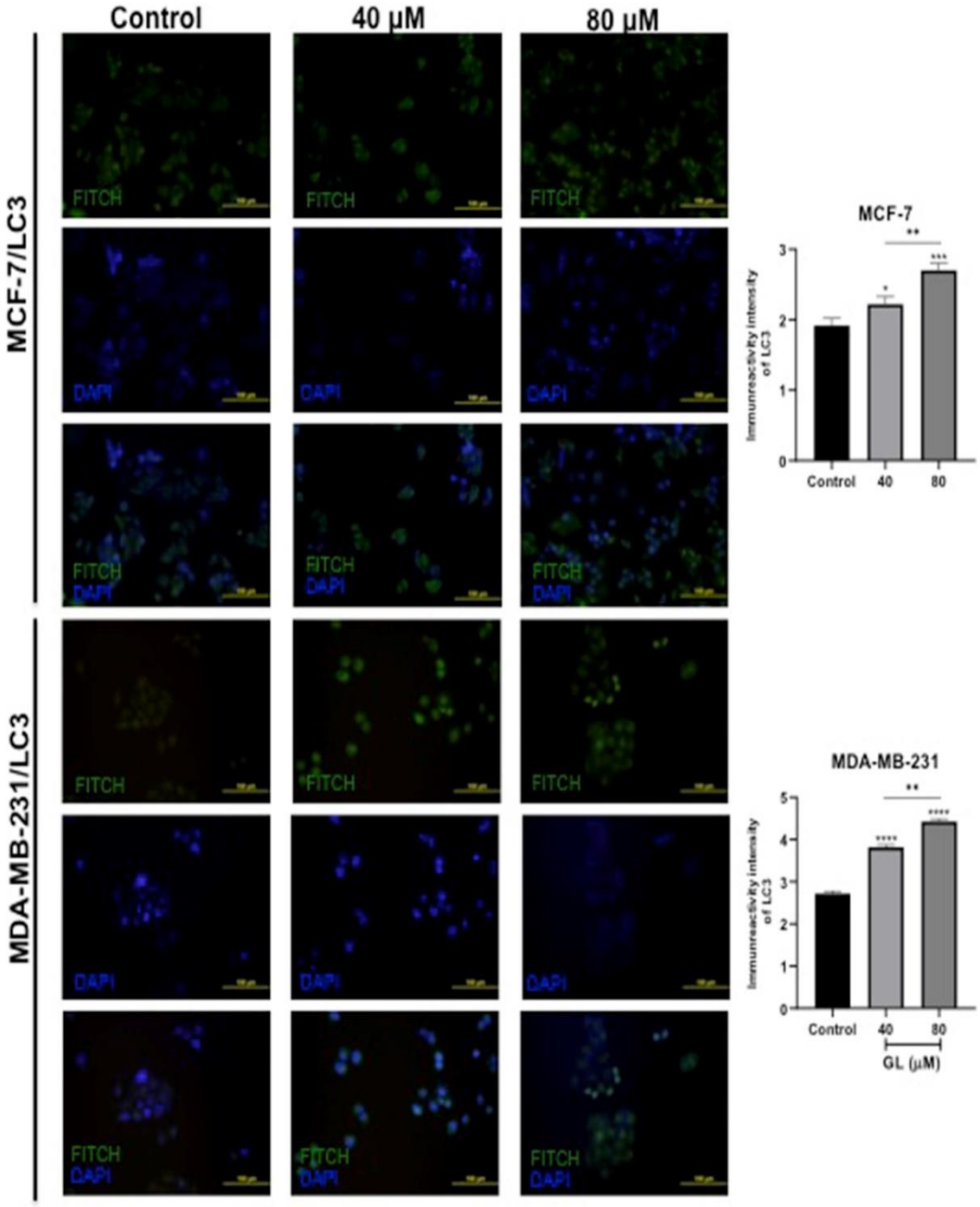

Figure 2. In the MCF-7 and MDA-MB-231 breast cancer cell line treated with GL, LC3 expressions in the groups are seen as green fluorescent reflections, Original magnification, ×400. Graphs show the effect of different doses GL on LC3 protein expression in MCF-7 and MDA-MB-231 breast cancer cells.

(Scale bar: µm, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

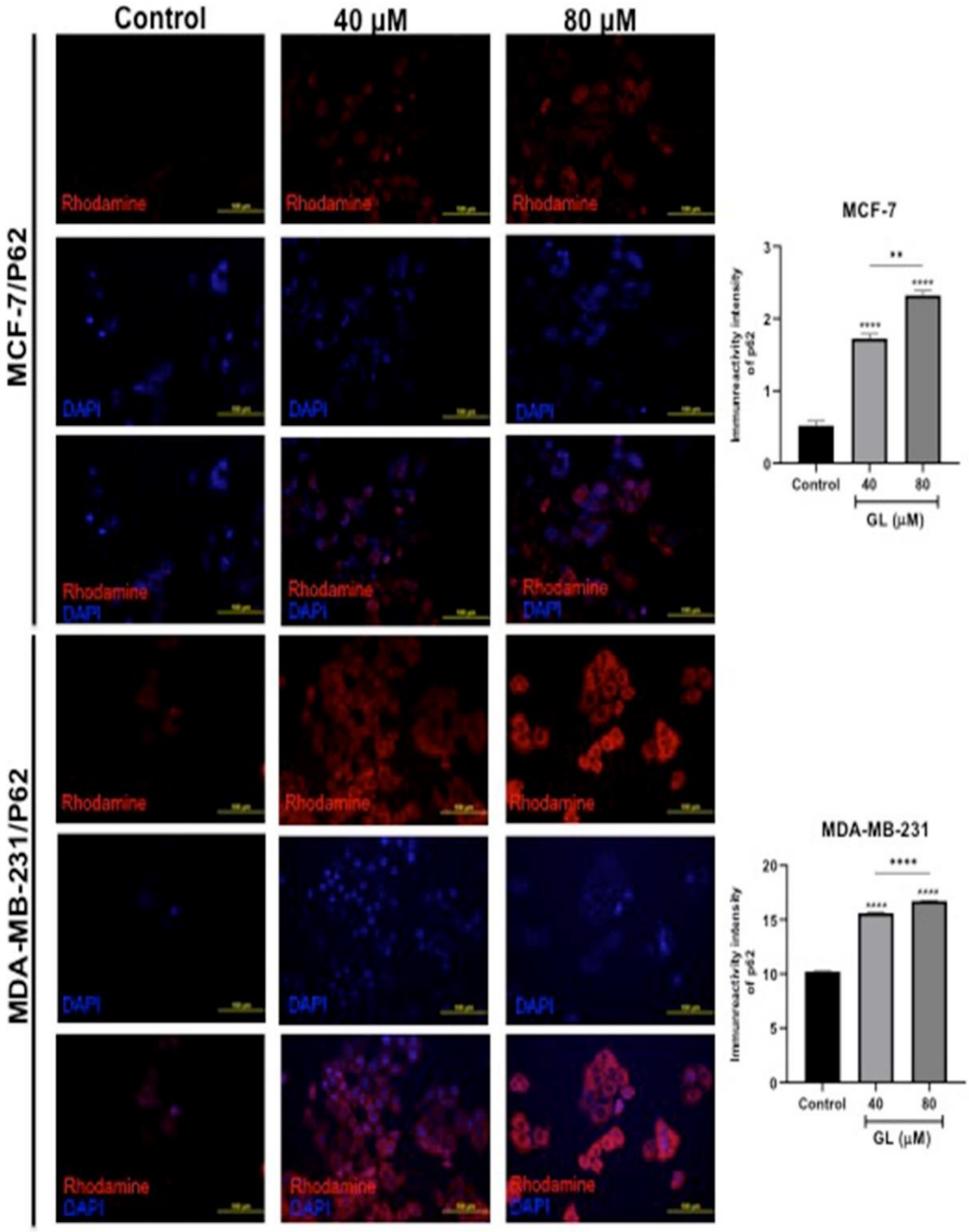

Figure 3. In the MCF-7 and MDA-MB-231 breast cancer cell line treated with GL, LC3 expressions in the groups are seen as red fluorescent reflections, Original magnification, ×400. Graphs show the effect of different doses GL on p62 protein expression in MCF-7 and MDA-MB-231 breast cancer cells.

(Scale bar: µm , **p<0.01, ****p<0.0001).

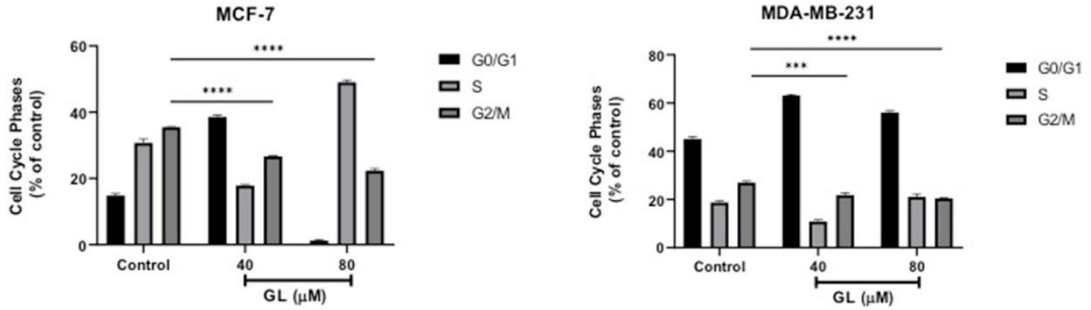

Figure 4. Cell cycle analysis. GL blocked cell division in the G2/M phase in both cell lines (***p<0.001, ****p<0.0001).

The effective doses were determined as 40 μM and 80 μM by MTT analysis in this study. This process was repeated three times, and the data obtained was combined and evaluated. Comparisons of percent viability values according to dose groups were made with one sample t test. This significant decrease clearly showed that GL dissolved in DMSO had a negative effect on cell viability and inhibition of proliferation.

Immunofluorescence demonstration of the effect of GL application on autophagy in MCF-7 and MDA-MB-231 breast cancer cell lines

By immunofluorescence staining, it was determined that Beclin-1 was expressed in both the nucleus and the cytoplasm inside the cell. In fluorescent microscope evaluations, red reflections in the cell cytoplasm indicated Beclin-1 expression. A significant difference was observed in Beclin-1 immunoreactivity intensity between the control and GL groups in both cell lines (respectively, $p<0.0001$, $p<0.0001$). There was also a significant difference between the 40 μM GL and 80 μM GL groups in the MCF-7 cell line ($p<0.0001$), but there was no significant difference between the 40 μM GL and 80 μM GL groups in the MDA-MB-231 cell line ($p>0.05$) (Figure 1). Beclin-1 measurements show that GL is more effective in MDA-MB-231 cells than MCF-7 at 40 μM.

LC3 expression was determined by the immunofluorescence staining method. It was determined that LC3 was expressed in the cell cytoplasm. In both cell lines, a significant difference was observed between the control and GL groups in terms of LC3 immunoreactivity intensity ($p < 0.05$, $p < 0.001$, $p < 0.0001$). In both cell lines, there was a significant increase between the 40 μM GL and 80 μM GL groups ($p<0.01$). According to LC-3 measurements, no significant difference was observed between MCF-7 and MDA-MB-231 cells. As indicated by green reflections in the cell cytoplasm in the evaluations made with a fluorescent microscope, LC3 expression is indicated (Figure 2).

The immunofluorescence staining method was used to determine p62 expression. It was determined that p62 was expressed in the cell cytoplasm. A significant difference was observed in p62 immunoreactivity intensity between the control and GL groups in both cell lines ($p < 0.0001$). It was determined that there was a significant increase between the 40 μM GL and 80 μM GL groups in both cell lines ($p<0.01$, $p<0.0001$). It was observed that GL had more effect on MDA-MB-231 cells than MCF-7 at 40 μm. The red reflections in the cell cytoplasm in the evaluations made with a fluorescent microscope indicated p62 expression (Figure 3).

Interpretation of cell cycle analysis

To investigate the effect of GL on cell division, cell cycle analysis was performed in GL-treated and untreated cells (control cells) using a cell cycle analysis kit suitable for the Muse device. The decrease in the G2/M phase in the experimental groups compared to the control group indicated that GL inhibited the cell cycle in the G2/M phase in both cell

lines ($p<0.0001$) (Figure 4). In line with this data, it was concluded that it induces autophagy by inhibiting the G2/M phase in the cell cycle.

DISCUSSION

Natural product compounds have received widespread attention for the prevention and treatment of cancer in recent years. Anticancer activities of plant-derived substances continue to be extensively studied at preclinical and clinical levels[24, 25]. Patients who receive cancer treatments such as chemotherapy and radiotherapy are exposed to serious side effects. Natural products have gained increasing attention as potentially non-toxic alternative agents for cancer treatments [24-26]. GL is a well-known traditional Chinese product that has been used in East Asia for over 2000 years to promote health and longevity[27]. Several studies have reported that GL polysaccharides (GLP) extracted from GL exert their anti-cancer activity through modulation of the immune system[28], inhibition of tumor cell proliferation, induction of tumor cell apoptosis[29], and suppression of drug resistance[30]. In light of all the promising results obtained, further research is required to explore the therapeutic potential of medicinal mushrooms and stimulate the development of drugs. As a result, the goal of this study was to see how GL affected cancer cell proliferation and autophagy.

In recent years, autophagy has been defined as a pathway that can be therapeutically targeted in cancer research. For the treatment of many diseases, identification of the molecular components of autophagy and its mechanism of action has been promising for the efficient development of therapeutics targeting autophagy [31, 32]. Many researchers have attracted attention by understanding this complex process and its modulation for cancer treatment. Pan et al. injected male BALB/C nude mice subcutaneously with HT-29 cells and subsequently treated them with *Ganoderma lucidum polysaccharide* (GLP) to determine the antitumor effect of GLP in vivo[18]. They reported that GLP treatment significantly reduced tumor volume compared with the control group, and the body weight of the mice was not affected by the GLP treatment. In the same study, they discovered that GLP increased LC3, p62, and p-MAPK/ERK levels in xenograft tumors while decreasing Beclin-1 and p-AMPKa levels[18]. In this study, LC3 and p62 levels were found to be higher in the GL-treated group than in the control group, which is consistent with the results of this study. Unlike in this study, Beclin-1 expression was found to be higher in the groups treated with GL, and the result was associated with the 24-hour period of the findings. In the next studies, the changes in the 48-hour and 72-hour periods were targeted. Our findings showed that GL induced dose-dependent autophagy in MCF-7 and MDA-MB-231 cells at these concentrations. In line with these data, when MCF-7 and MDA-MB-231 cells were compared in terms of autophagy markers, it was thought that MDA-MB-231 cells were more affected than MCF-7 cells.

Recent studies argue that cancer cells' response to therapies is mostly via cell death mechanisms such as cell cycle arrest, apoptosis, and autophagy[33-35]. The cell cycle consists of a series of events that allow cells to grow and reproduce. This process is completed with four successive phases called G1, S, G2, and M. The disruption that occurs in the cell cycle mostly contributes to the formation of cancer, so regulating cell cycle progression is one of the essential targets for anticancer therapies[35, 36]. Hu et al. showed that GL extracts inhibited the cell cycle of MCF-7 breast cancer cell lines in the G1 phase [37]. Similarly, Wu et al. reported that GL causes apoptosis in breast cancer cell lines by arresting the cell cycle in the G0/G1 phase [38]. In this study, it was shown that GL triggers autophagy by arresting the cell cycle in the G2 phase in MCF-7 and MDA-MB-231 breast cancer cell lines.

This study has potential limitations. First, the study was performed in vitro; the response of the same mechanism in vivo is unknown. Second, part of the autophagy pathway has been evaluated, and further evaluation is planned for future studies. In addition to immunofluorescence examination, it is planned to add methods at the molecular level.

In conclusion we found that GL treatment arrested the cell cycle progression at the G2/M phase in both breast cancer cell lines, dose-dependently. Based on our findings, GL has anti-proliferative activity by arresting the cell cycle at the G2/M phase and accelerates cell death by inducing autophagy, particularly in aggressive breast cancers that are resistant to treatment. In this way, it was concluded that GL can be evaluated as an alternative therapeutic agent, especially in the treatment of patients with resistant and aggressive breast cancer. However, further research is needed to determine the beneficial components of GL and whether they can be used as drugs in the future.

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