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Original Article
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Cannabigerol Treatment Shows Antiproliferative Activity and Causes Apoptosis of Human Colorectal Cancer Cells

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Objectives: To determine growth inhibitory and anti-cancer effects of Cannabigerol (CBG) in human colorectal cancer cells.
Methods: Anti-proliferative effect of CBG was examined using MTT assay and two colorectal cancer cells (SW480 and LoVo cells). Cell death ratio was analyzed using Annexin V/PI staining experiment. Cell cycle distribution was analyzed using flow cytometry. We also performed western blot analysis on apoptotic marker proteins.
Results: CBG showed growth inhibitory effect in colorectal cancer cells using MTT assay. IC_{50} concentration of CBG was 34.89 μM in SW480 cells and 23.51 μM in LoVo cells. Annexin V/PI staining showed that CBG treatment increased apoptotic cells from 4.8% to 31.7% in SW480 cells and from 7.7% to 33.9% in LoVo cells. Flow cytometry confirmed that CBG increased sub G_1 population via G_1 arrest in both SW480 and LoVo cells. Western blot analysis showed that CBG increased expression levels of cell death-related proteins such as cleaved PARP-1, cleaved caspase 9, p53, and caspase 3.
Conclusion: CBG treatment shows antiproliferative activity and causes apoptosis of colorectal cancer cells, suggesting that CBG is applicable as a promising anticancer drug.

Keywords: phytocannabinoid, cannabigerol, IC_{50}, colorectal cancer, apoptosis

INTRODUCTION

Colorectal cancer (CRC), characterized by aberrant proliferation of glandular epithelial cells in the colon or rectum, is the second most fatal cancer in the world [1, 2]. The major treatment modality for CRC is surgery, typically followed by chemotherapy [3]. The 5-year survival rate of CRC patients depends on the stage of CRC progression. If the cancer has metastasized, only about 10% of the patients survive beyond 5 years [4, 5]. 5-Fluorouracil (5-FU), an inhibitor of DNA synthesis, is the most widely used chemotherapeutic drug for CRC [6, 7]. The angiogenesis inhibitors bevacizumab (Avastin) and ramucirumab (Cyramza) also find place in CRC treatment. However, these drugs cause substantial side effects [8]. Currently, a variety of therapeutic strategies including chemotherapy agents are utilized for CRC treatment. In addition, many studies have been performed to identify new compounds from natural plant extracts with anticancer activities.

Cannabigerol (CBG) is a phytocannabinoid extracted from *Cannabis sativa* L. Phytocannabinoids are classified into over 113 types including CBG, cannabichromene (CBC), cannabidiol (CBD), Δ^9-tetrahydrocannabinol (Δ^9-THC), and cannabinol [9, 10]. CBG is synthesized non-enzymatically by decarboxylating cannabigerolic acid (CBGA), the precursor molecule of other cannabinoids [11]. CBGA can also be converted into acidic forms of THC, CBD, and CBC using three different

enzymes: tetrahydrocannabinolic acid synthase, cannabidiolic acid synthase, and cannabichromeric acid synthase, respectively [12, 13]. CBG is a minor component of approximately 10% of phytocannabinoids extracted from *C. sativa* [14]. These phytocannabinoids interact with the endocannabinoid system in humans and trigger signal transduction through cannabinoid receptor 1, cannabinoid receptor 2, serotonin 1A receptor, and transient receptor potential vanilloid 1 receptor (TRPV1) [15].

Given that CBG has no psychoactive effects, its therapeutic application in cancer is an interesting proposition [16]. CBG has been studied for its efficacy in neurological disorders, inflammatory diseases, and infections [17, 18]. CBG remains relatively understudied despite its unique chemical profile and potential therapeutic applications in cancer. CBG can prevent the progression of glioblastoma by activating the proapoptotic pathway [19]. CBG can also inhibit cell growth and colony formation and triggers cell cycle arrest. In addition, it was found to induce apoptosis in HuCC-1 and Mz-ChA-1 cholangiocarcinoma cells [20]. CBG can attenuate the production of colony-stimulating factor-1 (CSF-1) by melanoma cells, indicating that it functions as an anticancer agent by acting in the tumor microenvironment [21]. CBG was also found to reduce tumor progression in a xenograft model by lowering the number of tumor-associated macrophages [21].

Therefore, CBG seems to be a promising compound in cancer treatment. However, additional research is required to establish its effect using various cancer models. We performed a large array of molecular assays including the dimethylthiazole-2', 5'-diphenyl-2-H-tetrazlium bromide (MTT) assay, Annexin V/propidium iodide (PI) staining, fluorescence-activated cell sorting (FACS) analysis, and western blot analysis to clarify the effect of CBG against CRC cells. We found that CBG treatment reduces the proliferation and induces the apoptosis of human CRC cells.

MATERIALS AND METHODS

1. Cell culture and reagents

Human CRC cells LoVo and SW480 were purchased from Korea Cell Bank (Seoul, Korea). LoVo cells were maintained in RPMI-1640 (Welgene, Korea), and SW480 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Welgene) supplemented with 1X penicillin/streptomycin (LS 202-02; Welgene) and 10% fetal bovine serum (Merck Millipore, Germany). All cells were cultured in an incubator at 37℃ with 5% CO_2. CBG was purchased from Cayman Chemical (Ann Arbor, MI, USA) and dissolved in dimethyl sulfoxide (DMSO).

2. MTT assay

Cell viability was measured by the MTT assay. For this assay, MTT was diluted in phosphate-buffered saline (PBS). Cells were seeded into a 96-well plate at a cell density of 6×10^3 cells/well. After incubation overnight, the medium was replaced with DMEM containing various CBG concentrations ranging from 0 to 40 μM. The cells were incubated for up to 96 h. Every 24 h, 1X MTT solution was added to the 96-well plate and incubated for 4 h. The medium was removed, and purple formazan crystals were solubilized with 100 μL DMSO. The absorbance was measured at 570/690 nm wavelength using a microplate reader (Allsheng, China).

3. Annexin V/fluorescein isothiocyanate (FITC) staining

Cell death was analyzed using an Annexin V-FITC apoptosis detection kit (BioVision, Milpitas, CA, USA). SW480 and LoVo cells were seeded in 60 π culture dishes at a density of 6×10^5 cells/dish and incubated overnight. The SW480 and LoVo cells were treated with 30 μM CBG for 24 h to observe the fluorescence intensity. Cells were fixed with paraformaldehyde for 20 min. After fixing, the cells were incubated with 10 μL of PI and 5 μL of Annexin V for 10 min in the dark at room temperature. Annexin V was observed under a confocal microscope (Ts2, Nikon, Tokyo, Japan) at the Kangwon Center for System Imaging. To analyze the apoptotic cell population, the cells were washed twice with PBS and incubated with 10 μL of PI and 5 μL of Annexin V for 30 min in the dark at room temperature. Annexin V-FITC and PI fluorescence intensities were analyzed with a FACSymphony™ A3 Cell Analyzer (BD Bioscience, Franklin Lakes, NJ, USA).

4. FACS analysis and cell morphology

The concentration of CBG used for treatment was based on its IC_{50} values against LoVo and SW480 cells. After culturing for up to 48 h, the cells were harvested at 12 and 24-hour intervals and washed twice with PBS. The cells were fixed with 70% ethanol for 24 h. After fixing, the cells were stained with PI (50 μg/mL) for 30 min. Cell cycle distribution was analyzed

with a FACSymphony™ A3 Cell Analyzer (BD Bioscience). Cell morphology was imaged using a confocal microscope (Nikon, Eclipse TS100, Tokyo, Japan).

5. Western blot

Cells were seeded into 100 π dishes at a density of 6×10^6 cells/dish and treated with CBG for up to 48 h. Before lysis, the cells were washed once with PBS. Proteins were extracted using radio-immunoprecipitation assay buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with a protease inhibitor cocktail to extract total proteins. Protein concentrations were determined using Bradford reagent kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of proteins (40 μg) were electrophoresed on 12% and 8% gels and transferred onto a polyvinylidene fluoride (PVDF) membrane at 100 V for 1 h. The PVDF membrane was blocked using 5% skimmed milk for 30 min at room temperature, followed by incubation with specific primary antibodies at 4℃ overnight. Membranes were blotted with caspase 3, caspase 9, p53, poly (ADP-ribose) polymerase-1 (PARP-1), and β-actin primary antibodies. Afterward, the membrane was washed with 1X TBST buffer and incubated with secondary antibodies for 1 h at room temperature. Finally, the proteins were detected using an electrochemiluminescence kit (ATTO, Japan).

RESULTS

1. CBG inhibits cell growth and changes in cell morphology

We performed an MTT assay to analyze whether CBG could inhibit the growth of SW480 and LoVo cells (Fig. 1A). At concentrations below 10 μM, CBG did not affect cell growth. However, CBG at 20 μM inhibited the growth of both cell lines. No cell growth was observed in the presence of CBG at 40 μM. The IC_{50} value of CBG was estimated to be 34.89 μM for SW480 cells and 23.51 μM for LoVo cells. Therefore, the concentration of CBG was set as 30 μM for both cancer cells for further experiments. CBG-treated cancer cells demonstrated intracytoplasmic vesicles as a distinct time-dependent morphological change (Fig. 1B). Buoyant dead cancer cells were also noted in the CBG-treated group in a time-dependent manner. These results suggest that CBG can inhibit the growth and cause death of human CRC cells.

2. Annexin V is increased during cell death induced by CBG

Phytocannabinoids including CBD and THC can induce apoptosis in CRC cells. Therefore, we examined whether cell death induced by CBG was associated with an increased amount of Annexin V. We counted the number of dead cells using flow cytometry after Annexin V/PI staining (Fig. 2A, B).

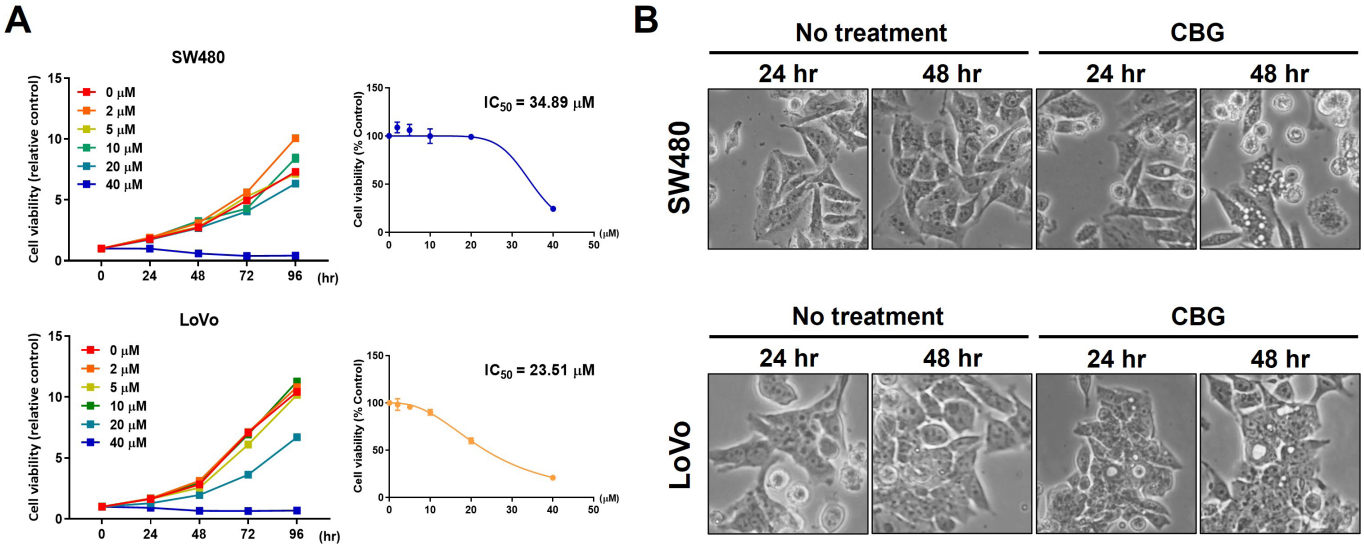

Figure 1. CBG inhibits cell growth and changes cell morphology. (A) Cell viability was determined using MTT assay for 96 hours. IC_{50} value of CBG was 34.89 μM for SW480 cells and 23.51 μM for LoVo cells. (B) Cell morphology was observed with a phase contrast microscope after CBG treatment. Dead cells and cytoplasmic vesicles were shown in CBG-treated cancer cells.

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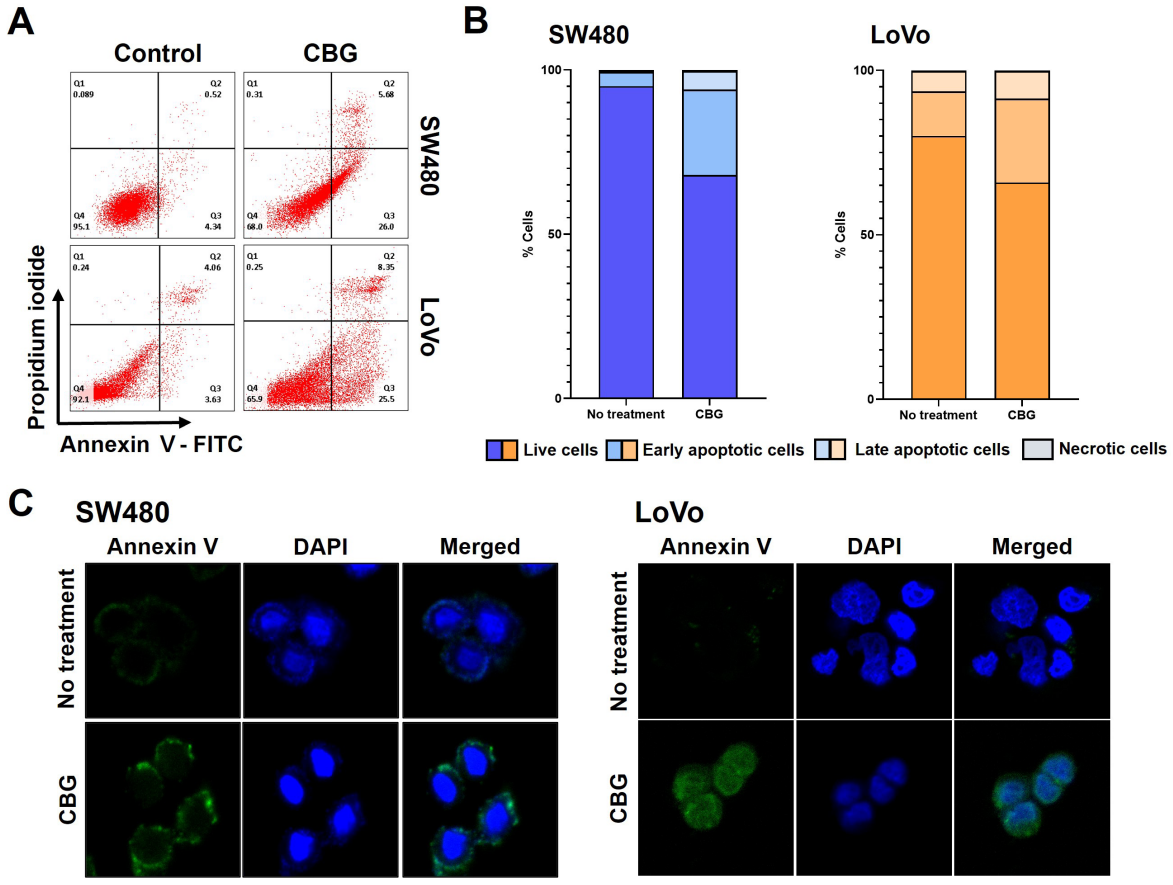

Figure 2. CBG treatment shows increased annexin V expression in colorectal cancer cells. (A) Colorectal cancer cells were treated with 30 μM of CBG for 24 hours. Apoptotic cells were analyzed with annexin V-FITC/PI staining using flow cytometry. (B) Graphs showing increased apoptotic colorectal cancer cells after CBG treatment. (C) Annexin V stained cells were observed with a confocal microscope after CBG treatment.

The proportion of apoptotic cell population increased after 24 h of CBG treatment. The proportion of early apoptotic cells increased 5.8-fold from 4.3% to 26% in SW480 cells and 7-fold from 3.6% to 25.5% in LoVo cells. The proportion of late apoptotic cells also increased by 5.2% from 0.5% to 5.7% in SW480 cells and increased by 4.3% from 4.1% to 8.4% in LoVo cells. When observed under a fluorescence microscope, the fluorescence intensity of Annexin V was observed near the cell membrane of both cancer cell lines after CBG treatment (Fig. 2C). These results suggest that CBG induces the typical cell death mechanism of phytocannabinoids in human CRC cells.

3. CBG treatment induces cell death via G_1 arrest

Time-dependent cell cycle distribution analysis after CBG treatment (Fig. 3) revealed that after 12 h of CBG exposure, the proportion of G_1 phase population increased from 55.9% to 75.7% in SW480 cells and from 53.6% to 62.6% in LoVo cells. The proportion of dead cell population increased to 38.4% in SW480 cells after 24 h of CBG exposure and 19.2% in LoVo cells after 48 h of CBG exposure. Therefore, CBG treatment altered cell cycle distribution by increasing cell death via G_1 arrest.

4. CBG treatment upregulates the expression of apoptotic marker proteins

To examine the mechanism by which CBG induces cell death, we examined the expression of cell death-related proteins by western blot analysis (Fig. 4A). CBG increased the expression of cleaved forms of caspase 9, PARP-1, and caspase 3, which are typical marker proteins for programmed cell death, in a time-dependent manner (Fig. 4B). These results indicate that CBG induces apoptosis of CRC cells.

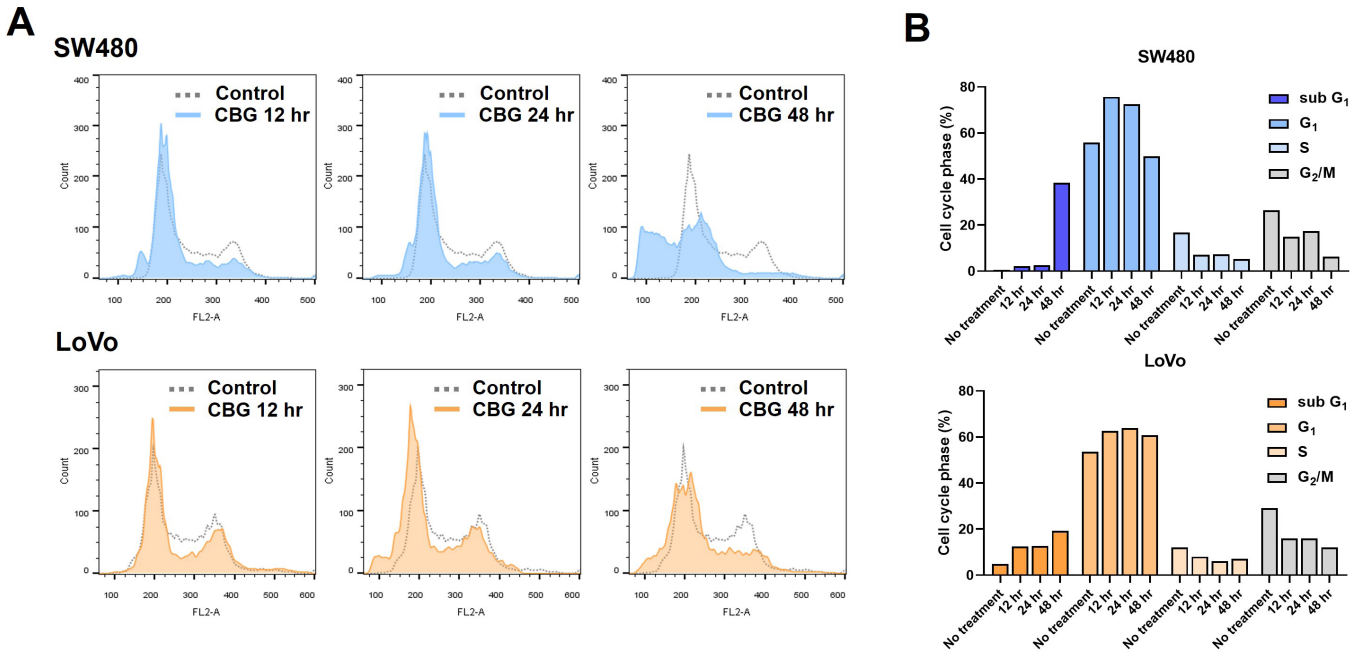

Figure 3. CBG treatment increases sub-G_1 population via G_1 arrest. (A) SW480 and LoVo cells were treated with 30 μM CBG for 48 hours. After that, cells were stained with propidium iodide for FACS analysis. (B) Graphs show percentage of each cell cycle distribution.

DISCUSSION

CBG is a non-psychoactive cannabinoid derived from *Cannabis sativa* [22, 23]. While CBG is present in lower concentrations in cannabis than other cannabinoids, it has been increasingly recognized for its therapeutic potential in many diseases including cancer [24, 25].

Its neuroprotective effect is similar to that of CBD. Studies on neural cell lines have shown that 2.5-10 μM CBG protects against hydrogen peroxide-induced neurotoxicity effects by reducing oxidative stress and mitochondrial dysfunction [26]. In the present study, we showed that CBG could inhibit cell growth, with IC_{50} values of 34.89 μM and 23.51 μM for SW480 and LoVo CRC cells, respectively. CBG was reported to reduce the viability of patient-derived primary glioblastoma cells at an IC_{50} of 100 μM [19]. In addition, CBG can inhibit cholangiocarcinoma growth at high concentrations of 100-200 μM [20]. Therefore, the IC_{50} of CBG depends on the cancer cell type. Our current study demonstrated that CBG inhibits the growth of two CRC cell lines at a concentration of 30 μM. Exposure at this concentration increased the proportion of buoyant dead cells, confirming the anticancer effects of CBG. Morphologically, we also observed vesicle formation in CBG-treated CRC cells. These morphological changes have also been observed previously in cancer cells treated with other cannabinoids. We previously demonstrated that CBD regulates intracellular vesicle formation in lung and colorectal cancers [27, 28]. Peroxisome proliferator-activated receptor γ (PPARγ), clathrin, and β-adaptin are known to regulate vesicle formation in lung cancer. In contrast, CBD induces the formation of vesicles in MCF7 breast cancer cells, suggesting a mechanism for cell death through autophagy [29]. CBD-induced vesicle formation might also depend on PINK1-Parkin-dependent mitophagy [30], suggesting that the formation of cytoplasmic vacuolation can occur through various mechanisms after cannabinoid treatment. Therefore, we see that CBG and CBD have different pharmacological effects in terms of morphological and functional changes, such as vesicle formation and cell death.

CBG is known to induce the apoptosis of many types of tumors, including prostate carcinoma, glioblastoma, and cholangiocarcinoma both *in vitro* and *in vivo* [19, 20, 31]. FACS analysis and Annexin V assay revealed that CBG induced cell death in CRC cells. At 30 μM, CBG increased the fluorescence of Annexin V in both cell lines. We also confirmed that CBG increased the levels of cleaved forms of PARP-1 and caspase 9. These data suggest that CBG could induce apoptotic cell death, making it a promising therapeutic drug for cancer.

Cannabinoids are known to improve prognosis and promote

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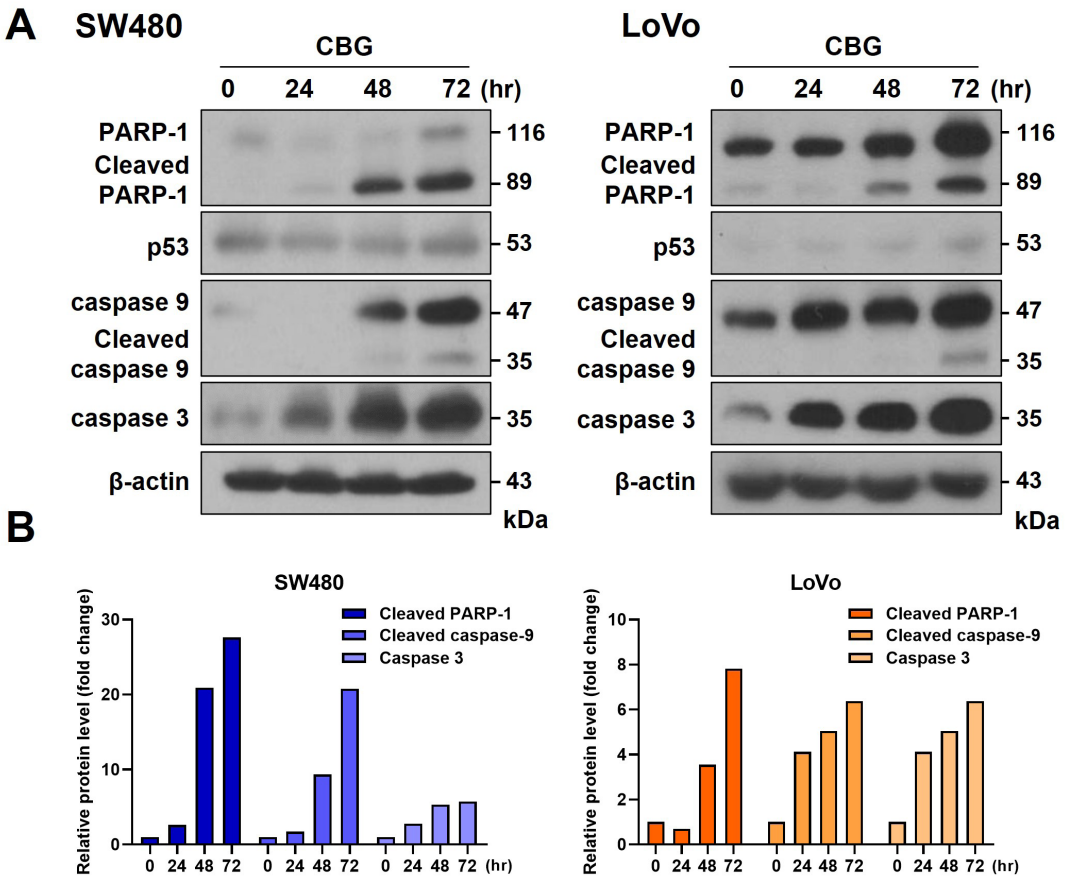

Figure 4. CBG treatment increases cleaved forms of caspase and PARP-1. (A) LoVo and SW480 cells were treated with 30 μM CBG, harvested at every 24 hours, and cultured for 72 hours. Western blot experiment was performed using PARP-1, caspase 9, caspase 3, and p53 antibodies. (B) Graphs showing increased intensities for cleaved forms of PARP-1, caspase 9, and caspase 3.

cancer regression in patients with different tumor types. A combination of curcumin, piperine, and cannabinoid variants was found to inhibit cell proliferation and induce apoptosis drastically in different CRC models [32]. Combination treatment of CBG and CBD exhibits the highest cytotoxicity via G-protein-coupled receptor 55 (GPR55) and TRPV1 signaling in glioblastoma stem cells, suggesting that CBG has potential in adjuvant standard-of-care therapy [19]. Exposure of ultraviolet A (UVA)-irradiated melanocytes to CBG significantly decreased the content of phosphatidylcholine, phosphatidylinositol, and sphingomyelin, which was increased by UVA, indicating that CBD and CBG can partially reverse the pro-cancerogenic changes in phospholipid profiles induced by UVA [33]. An optimal combination of CBG and CBD exhibited strong antitumor effects in mice with transgenic adenocarcinoma of the mouse prostate (i.e., TRAMP) by altering mitochondrial bioenergetics via voltage-dependent anion-selective channel 1, pointing to their therapeutical potential in prostate cancer [34]. A combination of THC, CBC, and CBG was demonstrated to exhibit synergistic effects with a PARP-1 inhibitor against ovarian cancer cells via the Wnt signaling pathway [35]. Therefore, cannabinoids may be used in combination to achieve synergistic anticancer effects.

CONCLUSION

Our results highlight the anticancer effects of CBG in two CRC cell lines. Although CBG seems to be a promising drug in preclinical cancer treatment, comprehensive studies are necessary to translate these findings into clinical applications. In addition, ongoing research into its mechanisms of action is crucial for developing effective CBG-based therapies for cancer.

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CONFLICTS OF INTEREST

Keun-Cheol Kim is an editorial board member of Journal of Pharmacopuncture but has no role in the decision to publish this article. No other potential conflicts of interest relevant to this aricle were reported.

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