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Combination of oxaliplatin and β-carotene suppresses colorectal cancer by regulating cell cycle, apoptosis, and cancer stemness *in vitro*

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ABSTRACT

BACKGROUND/OBJECTIVES: Colorectal cancer (CRC) is the third most common cancer worldwide with a high recurrence rate. Oxaliplatin (OXA) resistance is one of the major reasons hindering CRC therapy. β-Carotene (BC) is a provitamin A and is known to have antioxidant and anticancer effects. However, the combined effect of OXA and BC has not been investigated. Therefore, this study investigated the anticancer effects and mechanism of the combination of OXA and BC on CRC.
MATERIALS/METHODS: In the present study, the effects of the combination of OXA and BC on cell viability, cell cycle arrest, and cancer stemness were investigated using HCT116, HT29, OXA-resistant cells, and human CRC organoids.
RESULTS: The combination of OXA and BC enhanced apoptosis, G_2/M phase cell cycle arrest, and inhibited cancer cell survival in human CRC resistant cells and CRC organoids without toxicity in normal organoids. Cancer stem cell marker expression and self-replicating capacity were suppressed by combined treatment with OXA and BC. Moreover, this combined treatment upregulated apoptosis and the stem cell-related JAK/STAT signaling pathway.
CONCLUSIONS: Our results suggest a novel potential role of BC in reducing resistance to OXA, thereby enhances the anticancer effects of OXA. This enhancement is achieved through the regulation of cell cycle, apoptosis, and stemness in CRC.

Keywords: Beta-carotene; oxaliplatin; colorectal neoplasm; organoids; cell cycle

INTRODUCTION

Colorectal cancer (CRC) is the third most frequent malignant cancer, with an estimated 29,165 new CRC cases and 8,790 deaths expected in Korea in 2022 [1]. Despite significant advances in cytotoxic and targeted therapies, overcoming chemotherapy resistance remains a major obstacle. Therefore, there is a critical need to explore additional targeted therapies to improve the treatment of CRC [2].

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Conflict of Interest

The authors declare no potential conflicts of interests.

Author Contributions

Conceptualization: Kim Y; Formal analysis: Lee JH; Investigation: Lee JH, Kim Y; Methodology: Lee JH, Heo SC, Kim Y; Supervision: Kim Y; Writing - original draft: Lee JH; Writing - review & editing: Lee JH, Heo SC, Kim Y.

A recent study shows that the failure to improve survival after surgery combined with intraperitoneal chemotherapy following neoadjuvant chemotherapy may be due to the reduced efficacy of oxaliplatin (OXA) in patients whose residual cancer cells have developed resistance to both OXA and 5-flourouracil [3]. One of the main problems with the therapeutic use of platinum analogs is their intrinsic or extrinsic resistance to chemotherapeutic activity. Platinum-induced resistance is a multifactorial process involving alteration in transportation processes (uptake and efflux), resistance to DNA damage, apoptosis, and DNA repair [4]. Furthermore, there are still differences in the understanding of the factors contributing to platinum resistance and its application in clinical practice.

Cancer stem cells (CSCs) are a subset of cancer cells with the characteristics of self-renewal and tumor initiation and are resistant to chemical and electromagnetic stimulations [5]. The characteristics of these CSCs may explain the reason for cancer recurrence in patients after cancer treatment. The mechanisms of CSCs in enhancing drug resistances have been reported in recent trials [6,7]. Therefore, targeting CSCs to eliminate tumor cells and to prevent cancer recurrence is a good chemotherapy strategy.

The JAK-STAT signaling pathway is important in cytokine-mediated immune responses and is known to be involved in numerous biological processes such as stem cell regulation, apoptosis, migration, and proliferation. JAK/STAT is also frequently dysregulated in cancer cells. It has been reported that activation of JAK/STAT is positively associated with metastasis and shorter survival in CRC [8]. Inhibition of the JAK/STAT pathway was one of the mechanisms of anticancer therapy [9,10].

β-Carotene (BC), a provitamin A, is a carotenoid found in plant foods such as vegetables and fruits. The anti-cancer effects of BC are well-known. A combination of BC, vitamin E, and selenium exerted protective effects against gastrointestinal cancers in high-risk patients in China [11]. BC inhibited the growth of human CRC cells by regulating cell cycle arrest and apoptosis [12]. Furthermore, recent studies have demonstrated that BC may have an inhibitory effect on cell cycle progression, differentiation, cancer stemness, apoptosis, and cell proliferation in colon, breast, neuroblastoma, leukemia, and esophagus cancer cell lines [12-18]. However, the effect of combining BC with anticancer drug, particularly OXA, on CSCs and their underlying mechanisms have not yet been extensively studied. Furthermore, it is essential to explore the potential of BC in CRC treatment as an adjuvant therapy that is effective without causing side effects. Lastly, the use of more physiological model, such as human organoid, is necessary to better mick the human cell environment. In the present study, we examined the effect of BC and OXA combination and their mechanisms on CRC resistance using OXA-resistant HCT116 cells (HCT116/OXA) and human colon cancer organoids.

MATERIALS AND METHODS

Cell culture and reagents

Human colon cancer cells, HCT116 and HT29, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Fetal bovine serum (10%) (Gibco, Gaithersburg, MD, USA), penicillin (100 U/mL), and streptomycin (100 μg/mL) (Invitrogen, Carlsbad, CA, USA) were added into McCoy's 5A Medium (Welgene, Daegu, Korea) to maintain the cells. The cells were cultured at 37°C and 5% CO_2 in humidified conditions throughout the entire experiment. Due to its light sensitivity, BC (Sigma Aldrich, St. Louis, MO, USA) was liquefied

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with tetrahydrofuran under red light conditions. OXA (Sigma Aldrich) was dissolved using distilled water.

Establishment of OXA-resistant cell line

HCT116 cells were treated with increasing doses of OXA (Sigma Aldrich) for 4 weeks to produce stable OXA-resistant cells. The initial concentration of OXA was 0.5 μM, gradually increased up to the final dose of 5 μM. The established stable OXA-resistant HCT116/OXA cells were used for further experiments.

Cell viability assay in *in vitro* using cell lines and human colon organoids

MTT analysis was performed to determine the survival capacity of cells and organoids. HCT116, HT29, or HCT116/OXA cells were seeded in 96-well plates (2.5×10^3 cells/well). One day later, the cells were treated with IC_{50} doses of BC or OXA for 48 h. Subsequently, the medium was removed, and 200 μL of Thiazolyl 14 Blue Tetrazolium Bromide (MTT; Sigma Aldrich) solution was added to each well at 37°C for 4 h. Then, the supernatant was removed, and 100 μL of DMSO solution (Sigma Aldrich) was added to dissolve formazan crystals in each well. IC_{50} doses of BC or OXA in HCT116 were used for cell or organoid viability. Control group was treated with THF (0.125%). Absorbance values were measured at 570 nm using a microplate reader (Molecular Device, Sunnyvale, CA, USA). The percentage of absorbance values for each group was compared to a control group, where cell survival was considered to be 100%.

Cell cycle analysis

Flow cytometry, using BD Cycletest™ Plus DNA (Catalog No. 340242) (BD Biosciences, San Jose, CA, USA) was performed to assess the cell cycle and DNA content. The wavelength was set to 488 nm for Alexa fluor (BD Biosciences) fluorescence detection. The cell calculation rate for each cell cycle stage was assessed and set by the total number of cells, and the cell nuclei were analyzed by BD FACS Calibur™ (BD Biosciences).

Clonogenic analysis

Clonogenic analysis was performed to discover the self-renewal ability of colon CSCs [19]. Briefly, 200 or 300 cells/well of HCT116 or HCT116/OXA cells were seeded in 6-well-plates. OXA, BC, or their combination was then treated and cultured for 10–14 day. Thereafter, the colonies were fixed and dyed using a sodium chloride (NaCl) solution (0.9%) and crystal violet (Sigma Aldrich). The number of stained colonies was calculated as follows: (colony number/seeded cell number) × 100%.

Sphere formation assay

An additional analysis of the self-renewal capacity of CSCs is a sphere formation analysis [17]. HCT116 or HCT116/OXA cells (1×10^4 or 2×10^4 cells/well, respectively) were cultured in a 10% poly-(2-hydroxyethyl methacrylate) (Sigma Aldrich) coated 6-well plate. Basic fibroblast growth factor (40 ng/mL, Pepro Tech, London, UK), 2% of B27 (Invitrogen), and 20 ng/mL of epidermal growth factor (Pepro Tech) were added to DMEM-F12 (1:1; Welgene). After treating OXA or BC to cells for 8–10 day, the number of the spheres was counted and photographed using a phase-contrast microscope (Olympus, Tokyo, Japan).

Establishment of human colon organoid

Fresh human CRC and normal colon tissues were obtained from Seoul National University-Seoul Metropolitan Government Boramae Medical Center (Seoul, Korea). The human normal or colon tumor tissues were dissociated in a cold phosphate buffered saline (PBS;

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Stemcell Technologies, Vancouver, Canada) containing 2 mM EDTA (Sigma Aldrich), isolated, and maintained at a density of 2 – 2.5 × 10^5 cell/mL in Cultrex (BD Science) domes in a 24-well plate. The organoids were passaged and dissociated with 2 min TrypLE® Express (Thermo Fisher Scientific, Waltham, MA, USA) into single cells. The human normal colon organoids were grown using Intesticult organoid growth medium (Stemcell Technologies). The complete growth medium, Advanced DMEM:F12 (Sigma Aldrich), was added with 10 μM SB202190, 10 mM nicotinamide, 10 nM Gastrin, 1.25 mM N-acetyl cysteine, 50 ng/ml epidermal growth factor, 500 nM A83-01 (Sigma Aldrich), 2 mM Glutamax (Thermo Fisher), 100 ng/mL Noggin (Abcam, Cambridge, MA, USA), 1 × B-27 (Thermo Fisher), and 1 μ/mL primocin (InvivoGen, San Diego, CA, USA).

Immunofluorescence

For immunofluorescence staining, tumor colon organoids were treated with IC_{50} doses of HCT116 cells (OXA, 1.566 μM or BC, 32.51 μM) for 48 h, and then Cultrex was mechanically perturbed. After that, the organoid samples were isolated, infiltrated in PBS with 0.5% Triton X-100 for 90 min, and blocked in PBS with 3% goat serum for 2 h. The organoids were incubated with Lgr-5 and β-catenin (Abcam) overnight at 4°C and then incubated in Alexa Fluor 488-conjugated secondary antibody (Invitrogen) at room temperature (RT) for 1 h. The nucleus was stained with 25 μL of DAPI (Sigma Aldrich) for more than 30 min, and imaging was performed with a laser scanning microscope (LSM 880 Airyscan, Zeiss, Oberkochen, Germany).

Western blot analysis

A Western blot assay was performed as described earlier [20]. Proteins from HCT116, HT29, or HCT116/OXA cells treated with BC or OXA were extracted with RIPA lysis buffer. Each protein sample was transferred to a polyvinylidene fluoride membrane after being separated by electrophoresis using a sodium dodecyl sulfate-polyacrylamide gel. At RT, a 5% bovine serum albumin or a 5% fat-free skimmed milk solution was used to block the membranes for 1 h or more. Thereafter, the membranes were incubated with primary antibodies overnight at 4°C. The primary antibodies used in this study are as follows: CD133 (Miltenyi Biotec, Bergisch Gladbach, Germany), p-Jak1, Jak1, Cleaved caspase-3, p-Stat3, Stat3 (Cell Signaling, Danvers, MA, USA), and Bax, Bcl-2, Sox2, Oct4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Antibodies were visualized with an enhanced chemiluminescence test solution (Animal Genetics Inc. Suwon, Korea) after 3 times of washing. α-tubulin (Sigma Aldrich) was used as a loading control. The blots are quantified by the ImageJ software (v1.8.0; National Institutes of Health, Bethesda, MD, USA).

RNA preparation and quantitative real-time polymerase chain reaction (RT-PCR) analysis

RNA was extracted using a TRIzol reagent (Invitrogen). The cDNA was reverse transcribed using the RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific). After RT-PCR, the sample was combined with 2X SYBR Green PCR Master Mix (Hilden, Germany, Qiagen). The reaction was performed using a Rotor-Gene Q real-time cycler (Qiagen) under the following conditions; 5 min initiation at 95°C, 15 s denaturation at 95°C, and 10 s annealing and extension at 60°C. The primers sequences are as follows: 1) human glyceraldehyde 3-phosphate dehydrogenase (GAPDH): 5′- AGA AGG CTG GGG CTC ATT TG -3′ (forward) and 5′- AGG GGC CAT CCA CAG TCT TC -3′ (reverse); 2) human CD133: 5′- ATA CCT GCT ACG ACA GTC GT -3′ (forward) and 5′- TGG ATG CAG AAC TTG ACA AC -3′ (reverse); 3) human SOX2: 5′- CAA GAT GCA CAA CTC GGA GA -3′ (forward) and 5′- GCT TAG CCT CGT CGA TGA AC -3′ (reverse); 4) human OCT4: 5′- GTG AGA GGC AAC CTG GAG

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AA -3′ (forward) and 5′- GAA CCA CAC TCG GAC CAC AT -3′ (reverse). GAPDH was used as an internal control.

Statistical analysis

All results were presented as mean ± standard error of the mean (SEM), and *P*-values below 0.05 were considered statistically significant. All experiments were independently performed and analyzed using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA). The analysis was performed by one-way analysis of variance (ANOVA), followed by the Newman-Keuls post-test for multiple comparisons.

RESULTS

OXA, BC, or their combination significantly decrease cell viability of human CRC cells

To examine the effect of OXA or BC on cell viability, an MTT assay was carried out. HCT116, HT29, or HCT116/OXA cells were treated with OXA or BC at various doses for 48 h. OXA or BC significantly decreased cell viability of HCT116 (**Fig. 1A**), HT29 (**Fig. 1B**), and HCT116/OXA (**Fig. 1C**) cells in a dose-dependent manner.

Although IC_{50} values of BC showed no significant difference between the 2 cell lines, the IC_{50} value of OXA was significantly lower in HCT116 cells (1.57 μM) compared to that of HT29 cells (2.62 μM) (**Table 1**). Based on this result, HCT116 cells were used for further combination experiments and establishing the resistant cell line. HCT116/OXA cells showed significantly higher IC_{50} value of OXA (4.15 μM) than that of HCT116 cells (1.57 μM), which indicating that this HCT116/OXA cells possess stronger cancer resistance than HCT116 cells.

Cell growth was significantly decreased by OXA or BC treatment in HCT116, HT29, and HCT116/OXA cells. The combination treatment exerted more inhibition in cell growth compared to a single treatment of OXA or BC in all 3 cell lines (**Fig. 1D**). The combination of OXA and BC suppressed cell growth by approximately 71% ($P < 0.001$) in HCT116, 59% ($P < 0.001$) in HT29, and 62% ($P < 0.001$) in HCT116/OXA cells compared to the CTRL group. When HCT116/OXA cells were treated with the IC_{50} dose of HCT116 cells, cell viability was further suppressed by the combination treatment compared to OXA or BC alone. This indicates that resistance against OXA in HCT116/OXA cells was successfully established.

OXA, BC, or their combination induces G_2 phase cell cycle arrest in HCT116 and HCT116/OXA cells

A flow cytometric assay was carried out to analyze the effect of OXA, BC, or their combination on the cell cycle in HCT116 and HCT116/OXA cells (**Fig. 2**). G_1/G_0 phase was decreased in the

Table 1. IC_{50} value of OXA and BC in 3 cell lines

Compounds	IC_{50}		
	HCT116	HT29	HCT116/OXA
OXA (μM)	1.57[a]	2.62[b]	4.15[c]
BC (μM)	32.51[a]	32.70[a]	31.52[a]

The analysis was performed by 1-way analysis of variance, followed by the Newman-Keuls post-test for multiple comparisons. Alphabet letters are marked on the top to demonstrate that values are significantly different from each other ($P < 0.05$).
IC_{50}, half maximal inhibitory concentration; HCT116/OXA, oxaliplatin resistant HCT116 cell line; OXA, oxaliplatin; BC, β-carotene.

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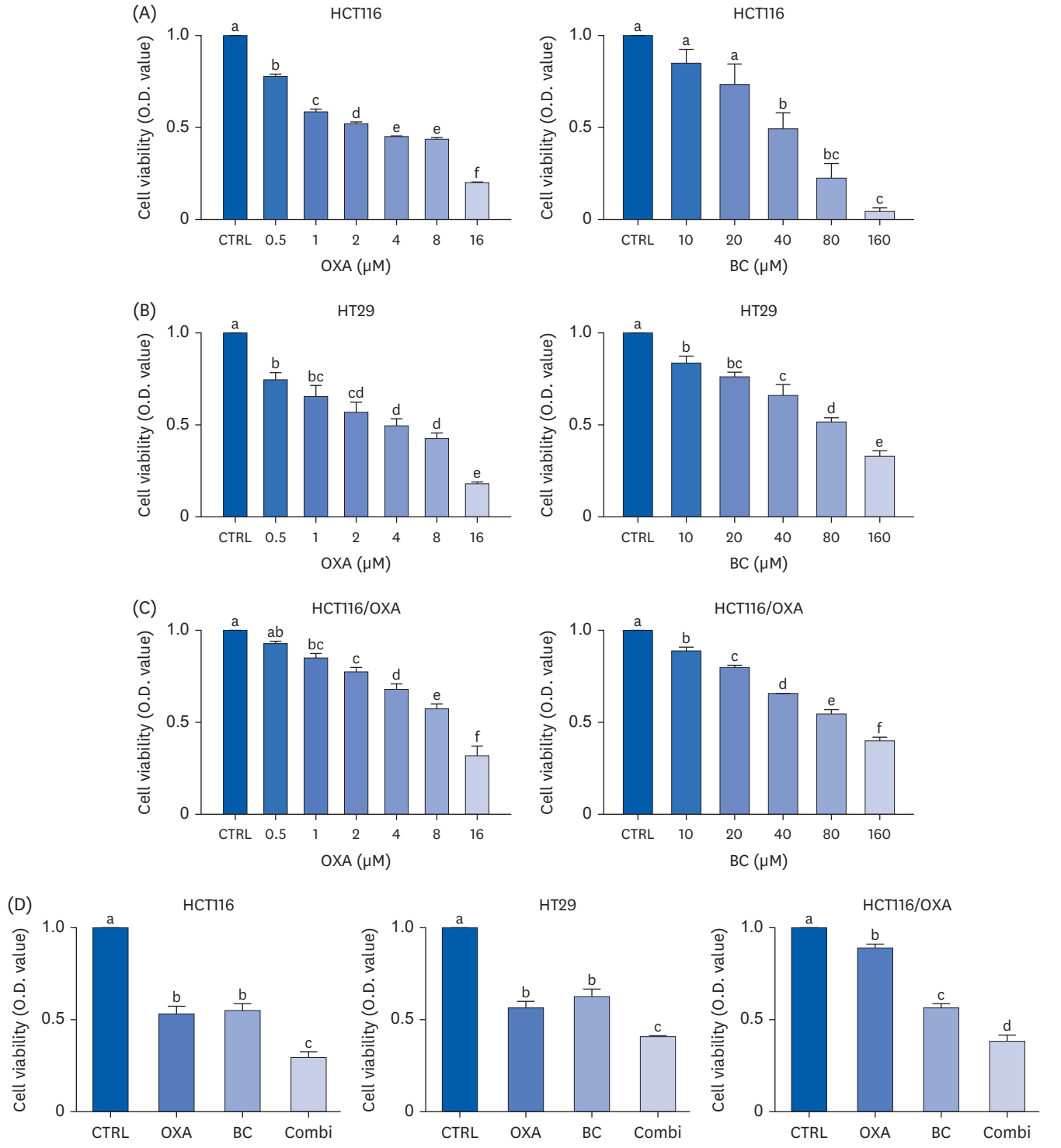

Fig. 1. OXA and BC decrease cell viability of HCT116, HT29, and HCT116/OXA cells. MTT assay was performed to assess cell viabilities of (A) HCT116, (B) HT29, and (C) HCT116/OXA. HCT116, HT29, and HCT116/OXA cells were treated with OXA (0, 0.5, 1, 2, 4, 8, and 16 μM) and BC (0, 10, 20, 40, 80, and 160 μM) for 48 h. (D) HCT116, HT29, HCT116/OXA cells were treated with IC_{50} doses of OXA (1.57 μM for HCT116, 2.62 μM for HT29, and 4.15 μM for HCT116/OXA), or BC (32.51 μM for HCT116, 32.70 μM for HT29, and 32.51 μM for HCT116/OXA) for 48 h. Data are presented as means ± SEM from at least 3 independent experiments. The analysis was performed by one-way analysis of variance, followed by the Newman-Keuls post-test for multiple comparisons. Statistically significant differences between groups are indicated by different alphabetical letters ($P < 0.05$).
O.D., optical density; CTRL, control; OXA, oxaliplatin; BC, β-carotene; Combi, combination; IC_{50}, half maximal inhibitory concentration.

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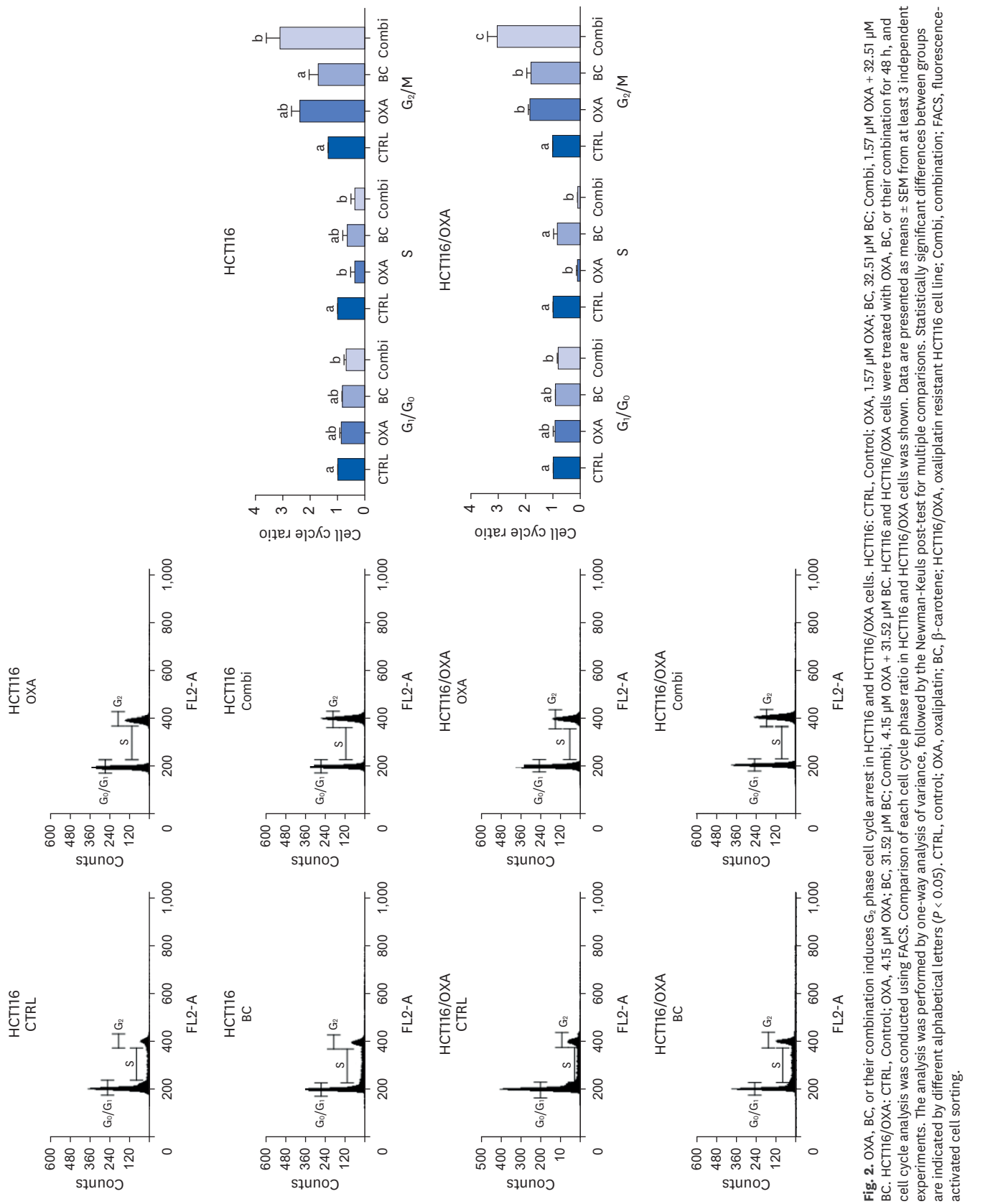

Fig. 2. OXA, BC, or their combination induces G_0 phase cell cycle arrest in HCT116 and HCT116/OXA cells. HCT116: CTRL, Control; OXA, 1.57 μM OXA; BC, 32.51 μM BC; Combi, 1.57 μM OXA + 32.51 μM BC. HCT116/OXA: CTRL, Control; OXA, 4.15 μM OXA; BC, 31.52 μM BC; Combi, 4.15 μM OXA + 31.52 μM BC. HCT116 and HCT116/OXA cells were treated with OXA, BC, or their combination for 48 h, and cell cycle analysis was conducted using FACS. Comparison of each cell cycle phase ratio in HCT116 and HCT116/OXA cells was shown. Data are presented as means ± SEM from at least 3 independent experiments. The analysis was performed by one-way analysis of variance, followed by the Newman-Keuls post-test for multiple comparisons. Statistically significant differences between groups are indicated by different alphabetical letters ($P < 0.05$). CTRL, control; OXA, oxaliplatin; BC, β-carotene; HCT116/OXA, oxaliplatin resistant HCT116 cell line; Combi, combination; FACS, fluorescence-activated cell sorting.

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Combi group to 30% ($P < 0.05$) in HCT116 cells and 20% ($P < 0.05$) in HCT116/OXA cells compared with the Ctrl group, whereas no significant difference was detected among other groups. The G_2/M phase ratio in HCT116 cells was significantly increased in the Combi group ($P < 0.05$) compared with the Ctrl group. The G_2/M phase ratio in HCT116/OXA cells was significantly increased by 1.8-fold by BC treatment, whereas it was increased by 3.0-fold in the Combi group ($P < 0.05$) compared to the Ctrl group in HCT116/OXA cells.

OXA, BC, or their combination induces apoptosis in human CRC cells and OXA-resistant HCT116/OXA cells

To determine the effects of OXA, BC, or their combination on apoptosis in human CRC cell lines, major apoptosis markers were analyzed using a Western blot assay (**Fig. 3**). After treating HCT116, HT29, and HCT116/OXA cells with OXA, BC, or their combination at various concentrations for 48 h, the expression of cleaved caspase-3 and Bax were significantly increased compared to the Ctrl group. Moreover, the Combi group showed the higher upregulation of cleaved caspase-3 and Bax levels compared to the Ctrl group. In HCT116 cells, the Combi group exhibited a 5.1-fold increase in cleaved caspase-3 expression ($P < 0.05$), while in HT29 cells, it showed a 2.2-fold increase ($P < 0.01$), and in HCT116/OXA cells, it showed a 4.2-fold increase ($P < 0.01$). Similarly, the expression of Bax in the Combi group was increased to 4.0-fold ($P < 0.05$) in HCT116 cells, 4.2-fold ($P < 0.05$) in HT29 cells, and 3.8-fold ($P < 0.05$) in HCT116/OXA cells compared to the CTRL group. As for Bcl2, its expression was downregulated the most in the Combi group, showing about an 81.2% decrease ($P < 0.001$) in HCT116 cells, 70% decrease ($P < 0.01$) in HT29 cells, and 78% decrease ($P < 0.001$) in HCT116/OXA cells. Taken together, the induction of apoptosis is one of the anti-CRC mechanisms of OXA, BC, or their combination, with the combination being more effective than individual treatments.

OXA, BC, or their combination decreases stemness of cancer cells

Self-renewal capacity is one of the characteristics of CSCs. To evaluate the effect of OXA, BC, or their combination on CSCs, a colony formation assay was conducted (**Fig. 4A**). After incubation with OXA, BC, or their combination to cells, the number of colonies in both HCT116 and HCT116/OXA cells was significantly decreased. The combination treatment significantly suppressed colony counts by approximately 90% ($P < 0.001$) compared to the CTRL group in both HCT116 cells and HCT116/OXA cells.

A sphere formation assay was performed to evaluate the self-renewal capacity of CRCs. After 48 h of OXA, BC, or their combination treatment, cancer cell spheroid formation was most inhibited in the Combi group compared to the CTRL group in both HCT116 and HCT116/OXA cells. The sphere sizes were reduced by approximately 68.7% ($P < 0.001$) in HCT116 cells and to 75.3% ($P < 0.001$) in HCT116/OXA cells in the Combi group (**Fig. 4B**).

The mRNA expression levels of cancer stemness markers, including *CD133*, *SOX2*, and *OCT4* were examined by RT-qPCR (**Fig. 4C**). In both HCT116 and HCT116/OXA cell lines, *CD133*, *SOX2*, and *OCT4* mRNA expressions were downregulated by OXA, BC, or their combination treatment. The most significant downregulation rate was observed in the Combi group for each cell line. The combination treatment led to a decrease in *CD133* level by 83% ($P < 0.001$), *SOX2* level by 72.7% ($P < 0.001$), and *OCT4* level by 60.7% ($P < 0.001$) in HCT116 cells. Similarly, in HCT116/OXA cells, the Combi group showed a decreased in *CD133* level by 87% ($P < 0.001$), *SOX2* level by 75% ($P < 0.001$), and *OCT4* level by 58% ($P < 0.001$). These results indicate that the addition of BC to OXA enhances the inhibition of the self-renewal capacity in CRC cells compared to each individual treatment.

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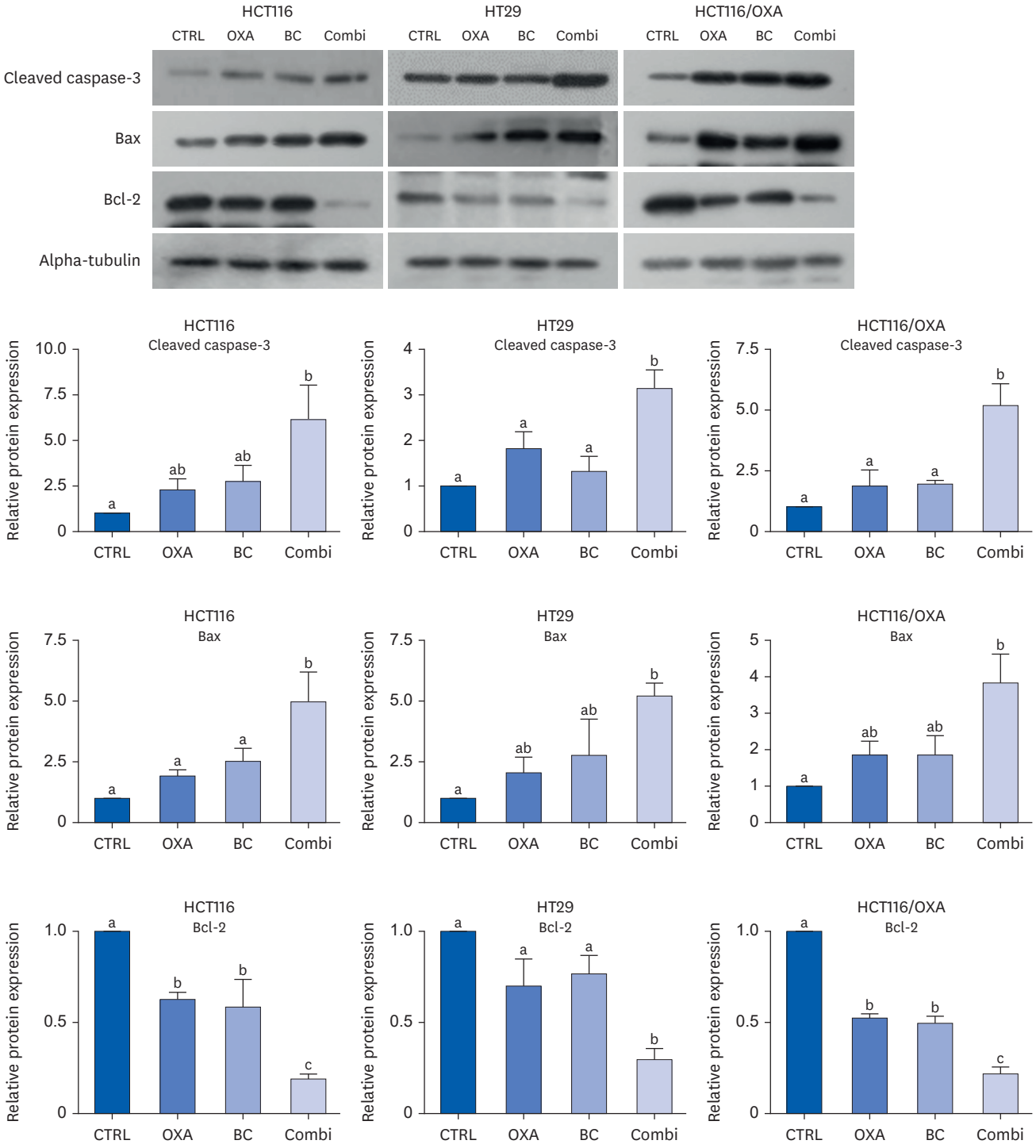

Fig. 3. OXA, BC, or their combination induces apoptosis in human CRC cells and OXA -resistant HCT116/OXA cells. HCT116: CTRL; OXA, 1.566 μM OXA; BC, 32.51 μM BC; Combi, 1.566 μM OXA + 32.51 μM BC. HT29: CTRL; OXA, 2.624 μM OXA; BC, 32.7 μM BC; Combi, 2.624 μM OXA + 32.7 μM BC. HCT116/OXA: CTRL; OXA, 4.15 μM OXA; BC, 31.52 μM BC; Combi, 4.15 μM OXA + 31.52 μM BC. Human CRC HCT116, HT29, and HCT116/OXA cells were treated with OXA, BC, or their combination for 48 h. Western blot assay was performed to detect the protein expressions of cleaved caspase-3, Bax, and Bcl-2, with alpha-tubulin as a loading control. The results are quantified by the ImageJ quantification program. Representative blots. Quantification of cleaved caspase-3, Bax, and Bcl-2 levels normalized to alpha-tubulin in HCT116, HT29, and HCT116/OXA cells are presented as means ± SEM from at least 3 independent experiments. The analysis was performed by 1-way analysis of variance, followed by the Newman-Keuls post-test for multiple comparisons. Statistically significant differences between groups are indicated by different alphabetical letters ($P < 0.05$).
CTRL, control; OXA, oxaliplatin; BC, β-carotene; Combi, combination; HCT116/OXA, oxaliplatin resistant HCT116 cell line; CRC, colorectal cancer.

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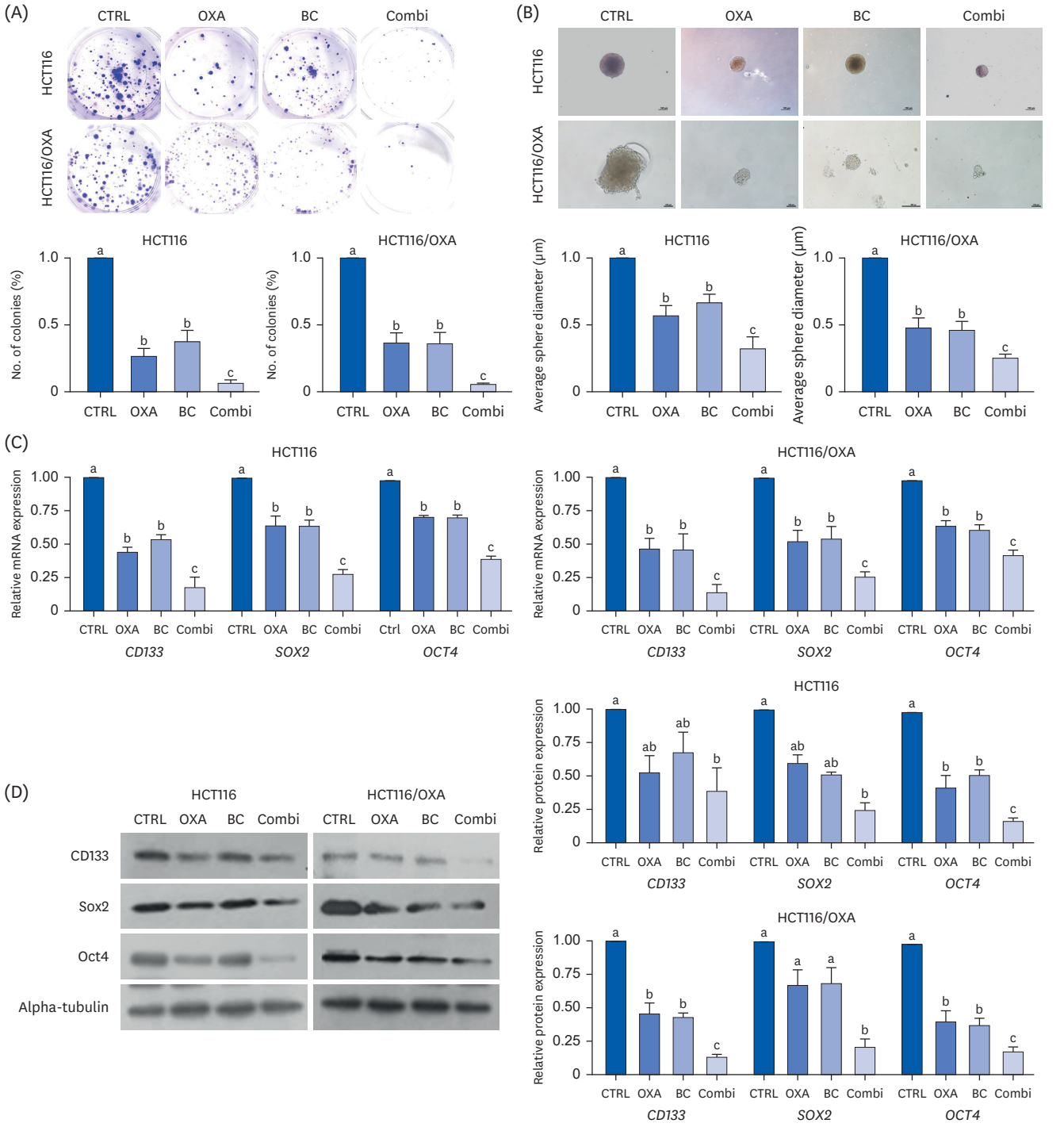

Fig. 4. OXA, BC, or their combination decreases stemness of cancer cells. HCT116: CTRL; OXA, 1.57 μM OXA; BC, 32.51 μM BC; Combi, 1.57 μM OXA + 32.51 μM BC. HCT116/OXA: CTRL; OXA, 4.15 μM OXA; BC, 31.52 μM BC; Combi, 4.15 μM OXA + 31.52 μM BC. (A) HCT116 and HCT116/OXA cells were treated with OXA, BC, or their combination in 6-well plates for 7–10 day. Colonies were stained and imaged. The number of colonies containing ≥ 50 cells was counted, and plating efficiency (percentage of colonies per input ± SEM) was calculated. (B) HCT116 and HCT116/OXA cells were cultured in sphere medium in poly-HEMA coated 6-well plates and treated with OXA and BC individually or in combination. Sphere formation was visualized (magnification, ×100). The number of spheres was counted, and (C) mRNA expressions of *CD133*, *SOX2*, and *OCT4* in HCT116 and HCT116/OXA cells were assessed by quantitative RT-PCR, with *GAPDH* as a loading control. (D) CD133, Sox2, Oct4, p-JAK1, JAK1, p-STAT3, and STAT3 protein expressions in HCT116 and HCT116/OXA cells were also analyzed by Western blot assay, with alpha-tubulin as a loading control. Representative Western blots are shown. Quantification graph of CD133, Sox2, and Oct4 levels normalized to alpha-tubulin in HCT116 cells and HCT116/OXA cells. The results are quantified by the ImageJ quantification program. Data are presented as means ± SEM from at least 3 independent experiments. The analysis was performed by one-way analysis of variance, followed by the Newman-Keuls post-test for multiple comparisons. Statistically significant differences between groups are indicated by different alphabetical letters ($P < 0.05$).
CTRL, control; OXA, oxaliplatin; BC, β-carotene; Combi, combination; HCT116/OXA, oxaliplatin resistant HCT116 cell line.

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To confirm the effect of OXA, BC, or their combination on the enriched cancer stemness of human CRC cells, a Western blot assay was performed (**Fig. 4D**). The protein levels of stemness markers CD133, Sox2, and Oct4 were examined. Consistent with the mRNA expression, CD133, Sox2, and Oct4 protein expressions were attenuated the most in the Combi group of HCT116 and HCT116/OXA cells. In the Combi group of HCT116 cells, the CD133 level was decreased by approximately 61.4% ($P < 0.05$), SOX2 level by 76.4% ($P < 0.001$), and OCT4 level by 82.8% ($P < 0.001$). Similarly, in the Combi group of HCT116/OXA cells, the CD133 level was decreased by approximately 87% ($P < 0.001$), SOX2 level by 79.4% ($P < 0.001$), and OCT4 level by 82.5% ($P < 0.001$).

JAK/STAT pathway is regulated in the anti-cancer effect of OXA, BC, or their combination in CRC.

The phosphorylation of Jak1 and Stat3 expressions was markedly decreased by OXA and BC, with the most significant downregulation observed in the Combi group in both cell lines (**Fig. 5**). In the Combi group, p-Jak1/Jak1 levels were decreased by approximately 72.3% ($P < 0.001$) in HCT116 cells and by 58.4% ($P < 0.001$) in HCT116/OXA cells. Similarly, p-Stat3/Stat3 levels in the Combi group were downregulated by approximately 86.7% ($P < 0.001$) in HCT116 cells and by 80% ($P < 0.001$) in HCT116/OXA cells compared to the Ctrl group. These results were associated with the anti-cancer stemness effect of OXA and BC, as the JAK1/STAT3 pathways is involved in cancer stemness.

OXA, BC, or their combination shows effectiveness in human colon organoid model.

To confirm the effect of OXA, BC, or their combination on more precise human-like model, cell viability assay and immunofluorescence was performed on human colon organoid model (**Fig. 6**). There was no significant change in cell viability in normal colon organoids treated with OXA, BC, or their combination compared to the CTRL group. However, significantly suppressed cell viability was detected in tumor colon organoids by OXA, BC, or their combination treatment. The combined treatment showed more suppression in cell viability compared with each treatment alone (**Fig. 6A**). These results indicate that OXA, BC, or their combination inhibits tumor growth without toxicity in normal tissues. In human normal colon organoids, no significant changes in size and number were detected in the Combi group compared with the Ctrl group. However, a marked reduction in size and number in the Combi group compared with the Ctrl group was found in tumor colon organoids (**Fig. 6B**). These results are confirmed with β-catenin, one of the organoid differentiation markers. β-Catenin expression was dramatically suppressed by the combined treatment (**Fig. 6C**). The results show that OXA and BC combination is more effective in reducing viability and tumor differentiation in human organoid model. These results provide future perspectives in the application of human organoid models to study the effectiveness of natural carotenoid in anticancer drug resistance.

DISCUSSION

This study demonstrated that the cell viability of human CRC cells and human tumor organoids was inhibited by the treatment of OXA, BC, or their combination. In addition, the combined treatment of OXA and BC downregulated the expression of apoptosis and cancer stemness markers in human CRC HCT116, HT29, and HCT116/OXA cells. Moreover, there was no significant change in cell viability, size, and number in normal colon organoids

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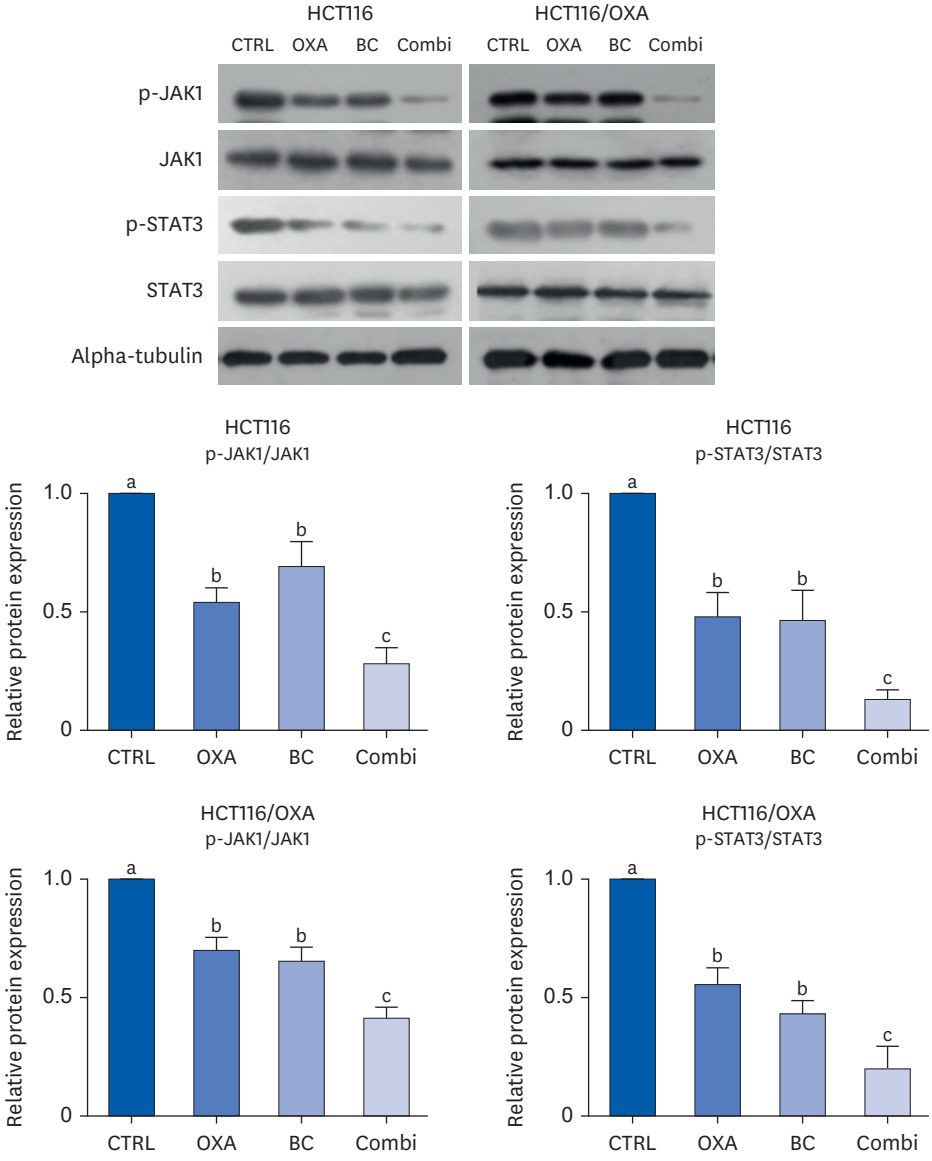

Fig. 5. JAK/STAT pathway is regulated in the anti-cancer effect of OXA, BC, or their combination in CRC.
HCT116: CTRL; OXA, 1.57 μM OXA; BC, 32.51 μM BC; Combi, 1.57 μM OXA + 32.51 μM BC. HCT116/OXA: CTRL; OXA, 4.15 μM OXA; BC, 31.52 μM BC; Combi, 4.15 μM OXA + 31.52 μM BC. Representative Western blots and relative quantification of p-JAK1 levels to JAK1 and p-STAT3 levels to STAT3 in HCT116 and HCT116/OXA cells were shown. The results are quantified by the ImageJ quantification program. Data are presented as means ± SEM from at least 3 independent experiments. The analysis was performed by one-way analysis of variance, followed by the Newman-Keuls post-test for multiple comparisons. Statistically significant differences between groups are indicated by different alphabetical letters ($P < 0.05$).
CTRL, control; OXA, oxaliplatin; BC, β-carotene; Combi, combination; HCT116/OXA, oxaliplatin resistant HCT116 cell line.

treated with OXA, BC, or their combination compared to the CTRL group. However, significantly suppressed cell viability, size, and number were detected in tumor colon organoids following the treatment with OXA, BC, or their combination.

Cancers are complex and individual-specific diseases, necessitating tailored strategies for personalized treatment to achieve the best clinical outcomes. Recently, organoid have emerged as a 3-dimensional *in vitro* culture system that can recreate the *in vivo* features of an individual's

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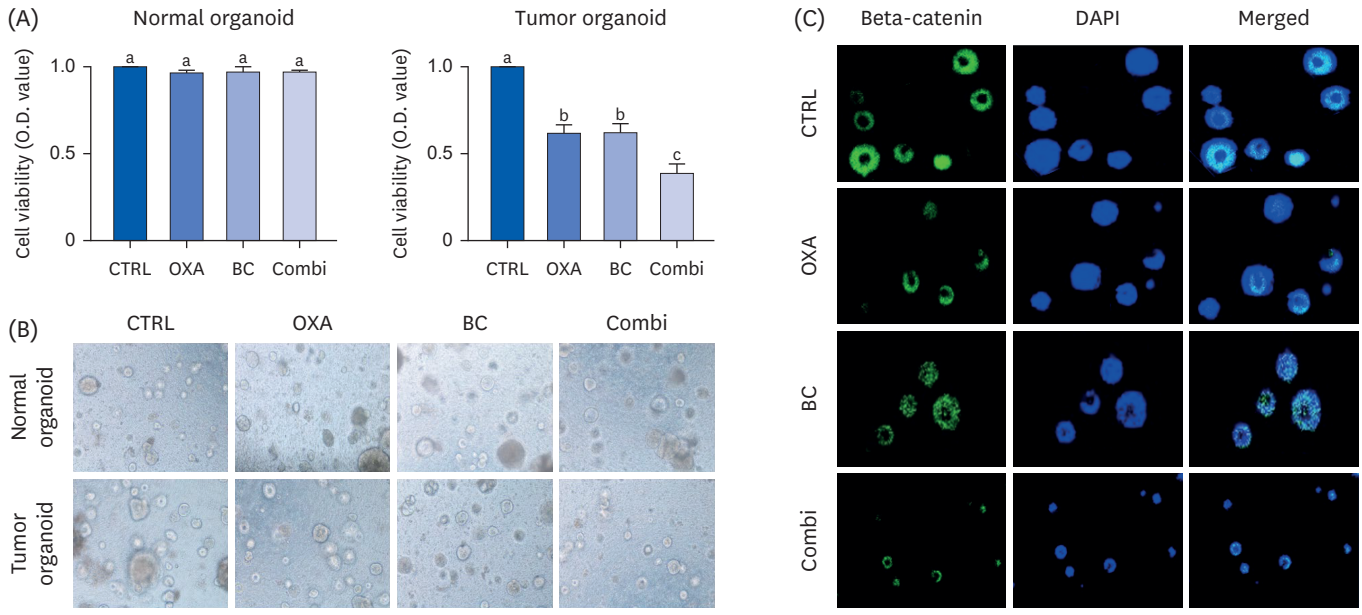

Fig. 6. OXA, BC, or their combination reduces cell viability and tumor differentiation in human colon organoids. Human colon organoids were treated with IC_{50} doses of OXA (1.57 μM), BC (32.51 μM), or Combi (OXA 1.57 μM and BC 32.51 μM) for 48 h. (A) Cell viability was assessed using the MTT assay. (B) The size and number of human normal and tumor organoids were measured and imaged using a phase-contrast microscope (magnification, ×100). (C) Immunofluorescence staining for beta-catenin (green) was performed on human colon tumor organoids (magnification, ×100). Data are presented as means ± SEM from at least 3 independent experiments. The analysis was performed by one-way analysis of variance, followed by the Newman-Keuls post-test for multiple comparisons. Statistically significant differences between groups are indicated by different alphabetical letters ($P < 0.05$). O.D., optical density; CTRL, control; BC, β-carotene; OXA, oxaliplatin; Combi, combination; IC_{50}, half maximal inhibitory concentration.

specific tissue. A previous study showed that a personalized patient-specific organoid proteome profile is highly correlated with disease diagnosis, offering the potential for the development of personalized therapies [21]. Moreover, human colon organoids can be used to identify normal stem/progenitor cells and exhibit discernible reactions to both normal and oncogenic Wnt signaling [22]. In the present study, the toxicity of BC and OXA treatment was evaluated using a more patient-specific approach with patient-derived tissue-oriented organoids.

In this study, OXA, BC, or their combination induced G_2 phase cell cycle arrest in HCT116 and HCT116/OXA cells. Previous studies have reported that OXA induces G_2/M phase cell cycle arrest in HCT116 cells [23], and BC gradually increases G_2/M phase cell distribution in LS-174 human colon adenocarcinoma cells [24]. Consistently, the combination treatment of OXA and BC in the present study induced apoptosis and G_2/M phase cell cycle arrest in HCT116/OXA cells more effectively than each treatment alone. These findings suggest that BC is an effective therapeutic approach in regulating resistance to OXA.

In the present study, OXA, BC, or their combination increased apoptosis in HCT116, HT29, and HCT116/OXA cells, with the combination showing the most effectiveness compared to each treatment alone. These findings align with previous studies that reported BC-induced cell cycle arrest and apoptosis in human CRC and myeloid leukemia [12,25]. Additionally, BC was observed to trigger apoptosis in MCF-7 breast cancer cells, leading to an increase in caspase 3-activity, while reducing the expression of anti-apoptotic markers, such as Bcl-2, PARP, and NF-kB survival proteins [26]. Recently, the use of drug combination therapies has become a growing trend to address side effects and combat chemoresistance [27]. These findings indicate that BC may possess potential anti-cancer combination therapy properties in cancer cells by promoting programmed cell death and inhibiting cell survival pathways.

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The results of this study show that the combination of OXA and BC significantly reduced sphere and colony formation, as well as the expression of stem cell markers in HCT116 and HCT116/OXA cells. Therefore, evaluating these 2 characteristics can determine the anticancer effect of a specific therapeutic agent on CSC. In the present study, the OXA and BC combination treatment inhibited the spherical formations of HCT116 and HCT116/OXA cells. Previous research has shown that BC inhibits the growth of several cancer cells, including colon CSCs [28]. In addition, it has been reported that BC suppressed the formation of colonies and spheres in both $CD133^+CD44^+$HCT116 and $CD133^+CD44^+$HT29 cells and downregulated protein and mRNA expression of cancer stemness markers in these CSCs [17,18]. Furthermore, we found that OXA, BC, or their combination inhibited cell growth in colon cancer organoids but did not exhibit toxicity to normal tissues, indicating the safety of this therapy. These results demonstrate that BC may hold potential as a therapeutic agent to overcome OXA resistance by inhibiting organoid viability and stem characteristics of human colon cancer organoids.

The effects of BC on JAK1/STAT3 activation were also evaluated in this study, and it was found that BC alone and in combination with OXA could attenuate JAK1 and STAT3 phosphorylation. The JAK/STAT pathway is critical in apoptosis, cell invasion, and migration in CRC [29]. STAT3 has been associated with chemotherapy and radiotherapy resistance, and STAT3 inhibitors are considered promising for cancer prevention and treatment [30]. Carotenoids are believed to prevent tumor cells growth through STAT3 inhibition. For example, picrocrocin, one of the saffron apocarotenoids, was found to inhibit STAT3 phosphorylation in SK-MEL-2 melanoma cells [31]. The antiapoptotic protein Bcl-2, a downstream target of STAT3, was also significantly suppressed by the combination of OXA and BC.

In conclusion, BC, OXA, or their combination inhibited OXA resistance by inhibiting apoptosis and cancer stemness while inducing cell cycle arrest. BC improved the anticancer effect of OXA and decreased OXA resistance through the JAK/STAT pathway. These results suggest that BC could serve as an additive to chemotherapeutic drugs, potentially applicable to other types of cancers as well.

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