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Orostachys japonica Induces Ferroptosis in 5-Fluorouracil-Resistantance Acquired SNU-C5 Colorectal Cancer Cells

Orostachys japonica Induce Ferroptosis en Células de Cáncer Colorrectal SNU-C5 Resistentes al 5-Fluorouracilo

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SUMMARY: Drug resistance is an un-met need in the treatment of colorectal cancer (CRC). Recently, the iron-dependent regulated cell death, ferroptosis, has been emerged as a potential therapeutic strategy for overcoming drug resistance in CRC. As natural products have been tested as promising candidates to induce ferroptosis in CRC, the anti-cancer effects of aqueous extract of *Orostachys japonica* (OJ) were investigated in 5-fluorouracil (5-FU)-resistance acquired SNU-C5 (SNU-C5/5-FUR) CRC cells in terms of ferroptosis. The cell viability, western blotting, and flow cytometry were used for analysis. OJ showed IC_{50} of 280 μg/μL *in vitro*. Long-term treatment of OJ (250 mg/kg) effectively induced ferroptosis with decreased GPX4, transferrin receptor, and ferroportin, and increased HMGB1 in SNU-C5/5-FUR cells-xenografted tissues. Short-term treatment of OJ (up to 250 μg/mL) did not show effective ferroptosis with slightly increased GPX4, transferrin receptor, and ferritin heavy chain. Co-treatment with ferroptosis inducers (RSL3 and sulfasalazine) or inhibitors (deferoxamine mesylate and ferrostatin-1) did not show any additive or synergistic effects on cell viability of SNU-C5/5-FUR cells. When co-treated with high dose deferoxamine mesylate, OJ further induced apoptosis as well as cell cycle arrest at G2/M phase. The results on ferroptosis *in vitro* and *in vivo* were inconsistent in SNU-C5/5-FUR cells, but ferroptosis *in vivo* indicating that other pathways might be a feasible candidate to induce ferroptosis or ferroptosis-augmented cell death in 5-FU resistant CRCs.

KEY WORDS: Ferroptosis; *Orostachys japonica*; Drug resistance; Colorectal cancer.

INTRODUCTION

Colorectal cancer (CRC) is well known as a common diagnosed cancer and a common cause of cancer-related death worldwide. Drug resistance still remains an un-met need in 5-fluorouracil (5-FU)-based chemotherapy of CRC, and thus, ferroptosis has emerged as a new potential therapeutic strategy for overcoming drug resistance in CRC (Cheng *et al*., 2023a). Ferroptosis is first introduced as a non-apoptotic and non-necrotic programmed cell death (Yang *et al*., 2014), in which glutathione peroxidase 4 (GPX4) is an essential regulator. Among 177 cancer cell lines tested, diffuse large B cell lymphoma and renal cell carcinoma are particularly susceptible to GPX4-regulated ferroptosis.

As the therapeutic effect of targeting ferroptosis is an active area of research in CRC (Cheng *et al*., 2023b; Zhang & Xie, 2024), natural products, based on their diverse bioactive activities and safety, have emerged as promising candidates to induce ferroptosis in CRC cells (Zhang & Xie, 2024). The key modulator proteins to ferroptosis are known as SLC7A11 (also known as system Xc- or xCT (cysteine/glutamate antiporter) which consists of SLC7A11 and SLC3A2) as well as GPX4. These modulators contribute to protecting cells from harmful reactive oxygen species. Meanwhile, iron metabolism also plays a central role in the process of ferroptosis (Estêvão *et al*., 2023; Zhang & Xie, 2024). Iron is an essential nutrient for several biological processes, including oxygen transport and cellular proliferation (Estêvão *et al*., 2023). In CRC, the balance between iron surplus and deficiency is paramount as it impacts tumorigenesis, progression, and drug resistance (Nelson, 1992, 2001). Elevated labile iron levels can also induce ferroptosis (Estêvão *et al*., 2023; Zhang & Xie, 2024), and thus, various regulators of iron metabolism, such as transferrin receptors (TfRC), ferritin, and SLC40A1 (ferroportin, FPN), intricately modulate ferroptosis. As a result, high mobility group box 1 (HMGB1), a damage-associated molecular pattern molecules (DAMPs), is released by ferroptotic cells (Wen *et al*., 2019).

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Orostachys japonica (OJ), known as Wa-song in the Republic of Korea, has been traditionally used a folk remedy to treat gastrointestinal cancers (Kim, 1984). To reveal the active ingredients of OJ extract, various solvents including ethanol, hexane, dichloromethane, ethyl acetate, n-butanol and water were tested. In the early days of the experiments, the ethyl acetate fraction of all the solvents showed the highest anti-cancer effects (Ryu *et al*., 2012). The ethyl acetate fraction of OJ showed cell cycle arrest and apoptosis in breast cancer (Kwon *et al*., 2019), pancreatic cancer (Kim *et al*., 2019), ovarian cancer (Lee *et al*., 2018), and gastric cancer (Ryu *et al*., 2012, 2014). Aqueous extract of OJ showed various biological properties including osteoclast de-differentiation (Shim *et al*., 2015), immunostimulation (Park *et al*., 2015), anti-proliferative effects as well as apoptosis in hepatic stellate cells (Kim *et al*., 2011), or anti-proliferative effects in CRC cells (Ryu *et al*., 2010; Kim *et al*., 2021).

Nevertheless, the effects of OJ on ferroptosis in CRC have yet to be thoroughly investigated, especially in drug resistance-acquired CRC. Chen *et al*. (2020) reported significant changes on ferroptosis-related proteins, in which GPX4, SLC7A11 (xCT), ferritin heavy chain (FTH1), and FPN were downregulated, but transferrin was upregulated in KRAS-mutant CRC cells. Park *et al*. (2022) revealed significant changes treated with fenbendazole, in which GPX4 and xCT were downregulated, but FTH1, FPN, and HMGB1 were upregulated in 5-FU-resistance acquired SNU-C5 (SNU-C5/5-FUR) CRC cells. Ferroptosis is also suggested as a potential anti-cancer mechanism *in vivo* model of CRC (Miao *et al*., 2023; Wang *et al*., 2024; Zou *et al*., 2024) with the known oral LD50 of higher than 2000 mg/kg/day of OJ in Balb/c mice (Kim *et al*., 2014). The anti-proliferative effects of OJ were observed in SNU-C5/5-FUR cells but apoptosis was not observed *in vivo* (Kim *et al*., 2021), and thus, there is a need for more diverse research in the field of cell death pathways. The present study aimed to investigate the ferroptosis on OJ-treated SNU-C5/5-FUR cells *in vitro* and *in vivo* based on the previous reports.

MATERIAL AND METHOD

Preparation of OJ. OJ was extracted with water as previously described (Kim *et al*., 2021). The lyophilized OJ was dissolved in distilled water just before experiments.

Reagents and antibodies. Reagents and antibodies used in this study were summarized in Table I.

Cell culture. SNU-C5/5-FUR cell line (Research Center for Resistant Cells; Chosun University, Gwangju, Republic of Korea) were cultured according to the supplier's protocol at 5 % CO_2, 37 °C, and humidified atmosphere conditions.

Cell viability assay. Cells were seeded in triplicate wells of 96-well plates, and treated with OJ (0, 62.5, 125, 250, and 500 μg/mL), RSL3 (0, 0.1, 1, 10, and 100 μM), sulfasalazine (SSZ; 0, 1, 10, 100, and 1000 μM), deferoxamine mesylate (DFOM; 0, 0.1, 1, 10, and 100 mM), and ferrostatin-1 (0, 0.1, 1, 10, and 100 μM) for 3 days. MTT solution was added for 3 h, and formazan crystals were dissolved in dimethylsulfoxide. The absorbance was read using VERSAmax microplate reader (Molecular Devices Korea LLC.; Seoul, Republic of Korea) as previously described (Kim *et al*., 2021; Yoon, 2023).

Subcutaneous tumor xenograft model. The animal samples of this study were athymic Balb/c nude mice that were also used in a previous study (Kim *et al*., 2021) with approval from the Jeju National University Institutional Animal Care and Use Committee (2019-0051). In brief, athymic Balb/c mice were subcutaneously injected with 1 x 10^6 SNU-C5/5-FUR cells, and then divided into 3 groups treated with distilled water (OJ0), 100 mg/kg (OJ100), and 250 mg/kg (OJ250) 3 times per week for 28 days. The

Table I. Reagents and antibodies used in the experiment.

	Name (catalog number)	Company	Purpose (action)
Reagent	MTT (M6494)	ThermoFisher Scientific (Seoul, Republic of Korea)	Cell viability
	Deferoxamine mesylate (DFOM; D9533)	Sigma-Aldrich (Merck KGaA, Darmstadt, Germany)	Ferroptosis inhibitor
	Ferrostatin-1 (SML0583)		Ferroptosis inhibitor
	Sulfasalazine (S0883)		Ferroptosis inducer
	RLS3 (B6095)	APExBIO (Houston, TX, USA)	Ferroptosis inducer
Antibody	GPX4 (ab125066)	Abcam (Cambridge, MA, USA)	Primary antibody
	SLC40A1 (Ferroportin, FPN; ab78066)		
	β-actin (sc-47778)	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	
	ferritin heavy chain (FTH1; sc-376594)		
	GAPDH (sc-47724)		
	Transferrin receptor (TfRC; sc-65882)		
	High mobility group box 1 (HMGB1; GTX62170)	GeneTex (Irvine, CA, USA)	
	SLC7A11 (xCT; ANT-111)	Alomone Labs (Jerusalem, Israel)	
	Anti-mouse IgG (PI-2000)	Vector Laboratories (Burlingame, CA, USA)	Secondary antibody
	Anti-rabbit IgG (PI-1000)		

resected tissue samples were used for Western blotting in this study.

Western blot analysis. Cells were treated with vehicle or OJ (100 and 250 μg/mL) for 1h or 3 days, followed by Western blot analysis as described previously. In brief, cell lysates (Yoon, 2023) and tissue samples (Kim *et al*., 2021) were subjected to gel electrophoresis with a polyvinylidene difluoride membrane. The membranes were incubated with primary antibodies and the appropriate secondary antibody. Protein bands were detected using the Azure™ c300 and quantified using the AzureSpot analysis software (version 14.2; Azure Biosystems Inc., Dublin, CA, USA).

Statistical analysis. All data were compiled from a minimum of 3 replicate experiments and are expressed as the mean ± SEM. A p-value of < 0.05, as determined using the Student's *t*-test or one-way analysis of variance followed by a post-hoc test (MS Excel, 2016), indicate a statistically significant difference.

RESULTS

OJ induces ferroptosis in SNU-C5/5-FUR xenografted tissues. The effects of OJ on cell viability were assessed using the MTT assay when treated with OJ for 3 days (Fig. 1A). The IC_{50} value of OJ on SNU-C5/5-FUR cell was 279.8 μg/mL. The cell viability was 87.2 ± 2.8 % (p = 0.001) and 72.3 ± 4.0 % (p < 0.001) at 125 and 250 μg/mL OJ, respectively.

Expression of the ferroptosis proteins was assessed by Western blot analysis when treated with OJ for 28 days (Fig. 1B and Table II). Compared with vehicle-treated condition, GPX4 (p < 0.001) was significantly decreased and HMGB1 (p = 0.002) increased. While FTH1 (p = 0.035) slightly increased, TfRC (p = 0.004) and FPN (p < 0.001) considerably decreased in OJ250 group.

OJ induces various responses of ferroptosis proteins in SNU-C5/5-FUR cells. Expression of the ferroptosis proteins was assessed by Western blot analysis when treated with OJ for 1 hour and 3 days, respectively, in SNU-C5/5-FUR cells.

1 hour after OJ treatment (Fig. 2A and Table II), dose-dependent increases were observed in xCT (p = 0.005), HMGB1 (p = 0.001), and FPN (p = 0.022) while GPX4 (p = 0.019), TfRC (p < 0.001), and FTH1 (p = 0.208) did not show dose-dependency. At 100 mg/mL OJ, GPX4 (p = 0.035) decreased and TfRC (p < 0.001) increased with significance.

3 days after OJ treatment (Fig. 2B and Table II), dose-dependent increase was observed in GPX4 (p = 0.023). HMGB1 (p = 0.050) did not show dose-dependency, of which HMGB1 (p = 0.002) significantly increased at 100 μg/mL OJ. xCT (p = 0.909), TfRC (p = 0.198), FTH1 (p = 0.097), and FPN (p = 0.355) did not show any considerable changes.

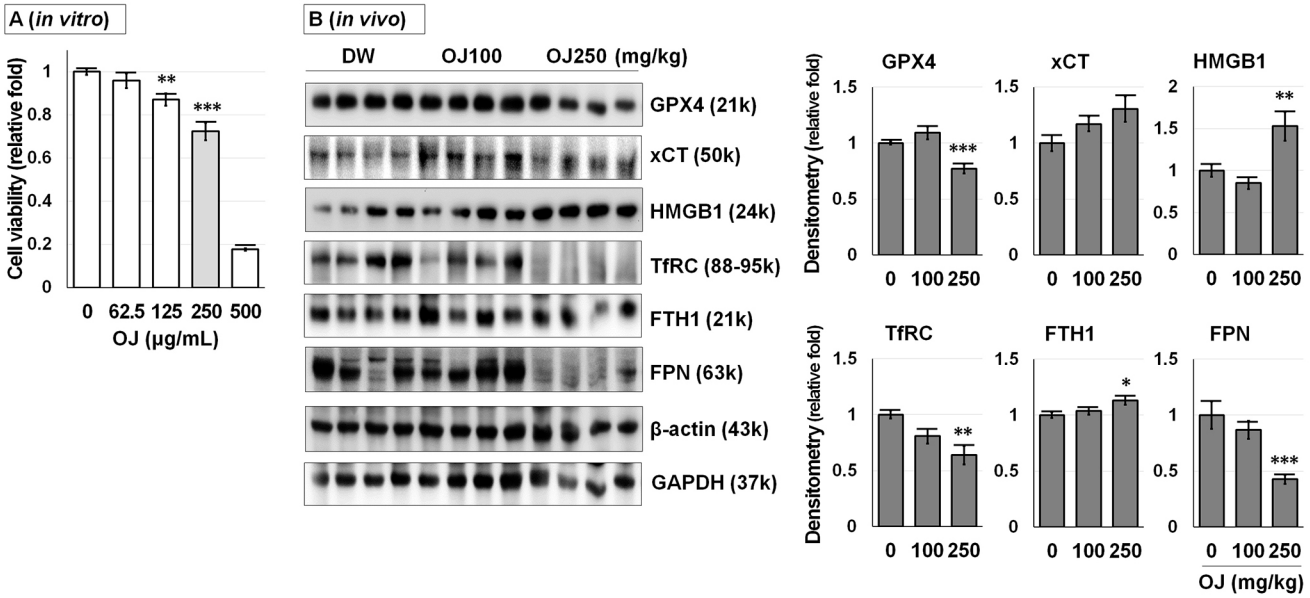

Fig. 1. *Orostachys japonica* (OJ) extract induces ferroptosis in SNU-C5/5-FUR cells-xenografted tissues: A. The cell viability was assessed by MTT assay in SNU-C5/5-FUR cells. OJ extract decreased the cell viability in a dose-dependent manner. B. Markers for ferroptosis were detected by immunoblotting while b-actin and GAPDH were used for loading controls in xenografted tissues. Expression levels of GPX4, TfRC, and FPN were significantly decreased, but HMGB1 significantly increased. *p < 0.05, **p < 0.01, ***p < 0.001 *vs.* vehicle-treated control.

Table II. Densitometric comparison on ferroptosis-related proteins after *Orostachys japonica* (OJ) extract treatment at various time points in SNU-C5/5-FUR cells. Results are expressed as relative fold when the vehicle-treated control was considered 1, respectively.

		***in vitro* (μg/mL)**				***in vivo* (mg/kg)**	
		1 hour	*p*-value	**3 days**	*p*-value	**28 days**	*p*-value
GPX4	OJ100	0.90 ± 0.03	0.035*	1.02 ± 0.07	0.468	1.09 ± 0.06	0.082
	OJ250	1.19 ± 0.10	0.063	1.39 ± 0.15	0.023*	0.77 ± 0.04	< 0.001***
xCT	OJ100	0.99 ± 0.06	0.370	0.95 ± 0.08	0.150	1.17 ± 0.06	0.050
	OJ250	1.21 ± 0.08	0.020*	0.98 ± 0.11	0.275	1.30 ± 0.11	0.026*
HMGB1	OJ100	1.52 ± 0.17	0.015*	1.47 ± 0.10	0.002**	0.85 ± 0.07	0.079
	OJ250	1.77 ± 0.12	< 0.001***	1.08 ± 0.20	0.396	1.53 ± 0.17	0.007**
TfRC	OJ100	1.38 ± 0.05	< 0.001***	1.20 ± 0.11	0.042*	0.81 ± 0.07	0.012*
	OJ250	1.14 ± 0.11	0.133	1.30 ± 0.15	0.021*	0.64 ± 0.18	0.001**
FTH1	OJ100	0.98 ± 0.03	0.194	1.00 ± 0.07	0.391	1.04 ± 0.03	0.190
	OJ250	1.12 ± 0.09	0.064	1.26 ± 0.14	0.060	1.13 ± 0.04	0.010*
FPN	OJ100	1.24 ± 0.05	0.001**	0.85 ± 0.05	< 0.001***	0.87 ± 0.08	0.190
	OJ250	1.28 ± 0.10	0.020*	0.95 ± 0.11	0.154	0.43 ± 0.04	0.001**

*p < 0.05, **p < 0.01, ***p < 0.001 vs. vehicle-treated control.

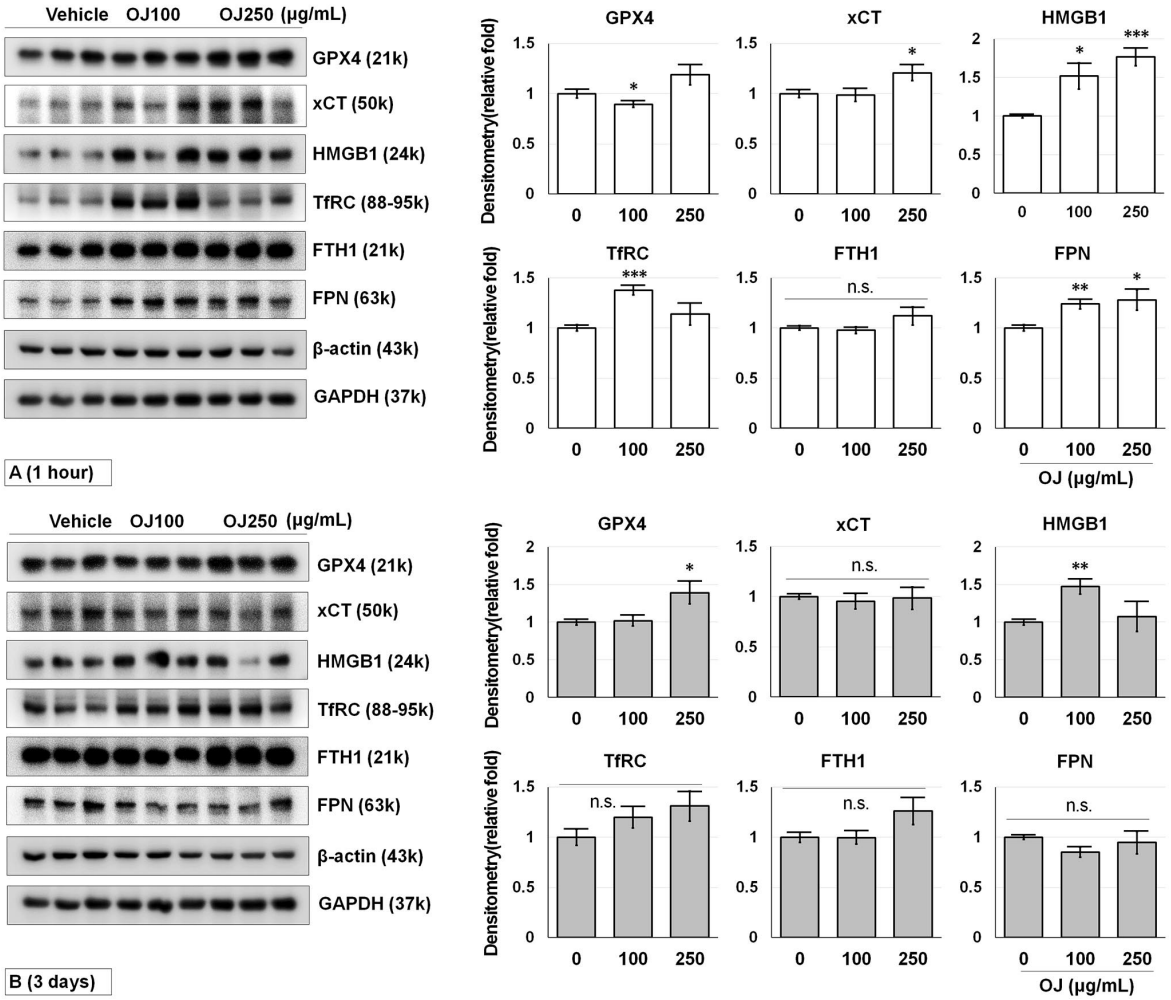

Fig. 2. *Orostachys japonica* (OJ) extract induces various responses of ferroptosis in SNU-C5/5-FUR cells: Markers for ferroptosis were detected by immunoblotting while β-actin and GAPDH were used for loading controls in SNU-C5/5-FUR cells. A. Expression levels of xCT, HMGB1, and FPN were significantly increased in a dose-dependent manner 1 hour after OJ treatment. B. Expression level of GPX4 was significantly increased, while other markers were not considerably changes 3 days after OJ treatment. *p < 0.05, **p < 0.01, ***p < 0.001 vs. vehicle-treated control; n.s. no significance

Ferroptosis inducer and inhibitor do not change the effects of OJ in SNU-C5/5-FUR cells. SNU-C5/5-FUR cells were incubated for 3 days with single chemicals and co-treated with OJ, then subjected to MTT assay. The effects of ferroptosis inducers, RSL3 (GPX4 inhibitor) and SSZ (xCT inhibitor), and ferroptosis inhibitors, DFOM (iron chelator) and Ferrostatin-1 (ferroptosis inhibitor) were assessed using the MTT assay. In case of single treatment (Fig. 3A), RSL3 decreased the cell viability in a dose-dependent manner, in which the survival rate of 43.2 ± 3.6 % at 1 μM. SSZ slightly increased the cell viability up to 100 μM, but decreased the survival rate of 35.2 ± 1.7 % at 1 mM. DFOM decreased the cell viability in a dose-dependent manner, in which the survival rate of 74.6 ± 2.1 % at 10 μM. Ferrostatin-1 slightly increased the cell viability up to 10 μM, but decreased the survival rate of 86.5 ± 3.7 % at 100 μM. When co-treated with OJ (Fig. 3B), RSL3 ($p < 0.001$) and SSZ ($p = 0.002$) dose-dependently decrease the cell viability but DFOM ($p = 0.598$) and ferrostatin-1 ($p = 0.255$) did not show any significant changes.

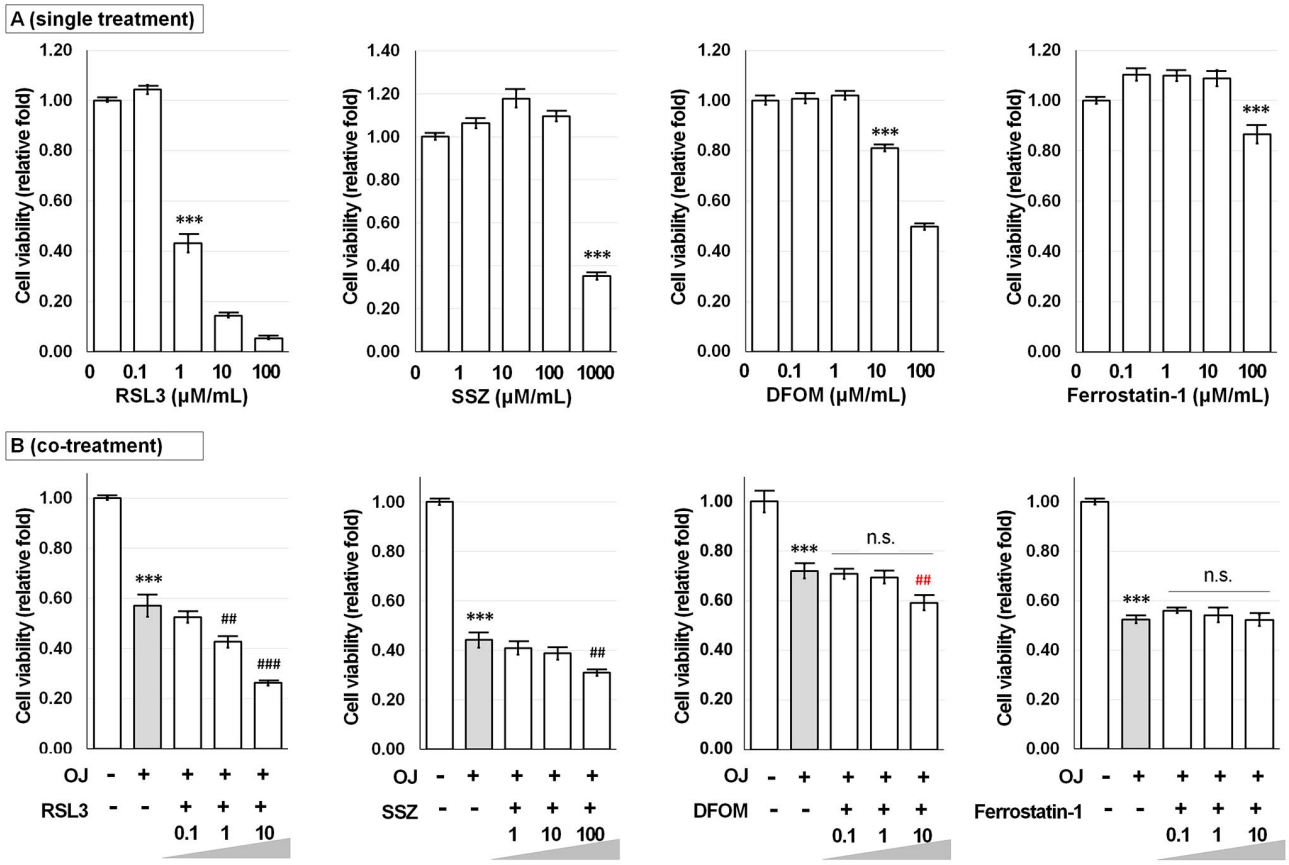

Fig. 3. Ferroptosis inducers and inhibitors do not change the effects of *Orostachys japonica* (OJ) in SNU-C5/5-FUR cells. The cell viability was assessed by MTT assay in SNU-C5/5-FUR cells in single treatment and co-treatment with OJ extract (250 μg/mL). A. Ferroptosis inducers (RSL3 and sulfasalazine (SSZ)) and inhibitors (deferoxamine mesylate (DFOM) and ferrostatin-1) decreased the cell viability. B. When co-treated with OJ, ferroptosis inducers dose-dependently decrease the cell viability but ferroptosis inhibitors did not show any significant changes. ***$p < 0.001$ *vs.* vehicle-treated control; ##$p < 0.01$, ###$p < 0.001$ *vs.* OJ; n.s. no significance.

Co-treatment with DFOM and OJ induces apoptosis as well as G2/M arrest in SNU-C5/5-FUR cells. SNU-C5/5-FUR cells were incubated for 3 days with vehicle, OJ (250 μg/mL), DFOM (10 μM), and OJ + DFOM (Fig. 4A), then subjected to flow cytometry (Fig. 4B).

Cell cycle analysis revealed a significant decrease in G0/G1 phase ($p < 0.001$) and an increase in G2/M phase ($p < 0.001$). The fraction of cells in G2/M phase demonstrated an upward trend in DFOM (47.9 ± 0.7 %, $p < 0.001$ *vs.* control, $p < 0.001$ *vs.* OJ) and OJ + DFOM (52.1 ± 0.8 %, $p < 0.001$ vs. control, $p < 0.001$ vs. OJ) groups when compared with untreated (24.5 ± 0.8 %) and OJ (25.4 ± 0.2 %)-treated conditions. The fraction of cells in S phase increased in DFOM (35.0 ± 0.6 %, $p < 0.001$ *vs.* control, $p < 0.001$ *vs.* OJ) but sustained in OJ + DFOM

(29.8 ± 0.6 %; p = 0.018 *vs.* OJ, p = 0.002 *vs*. DFOM) groups when compared with untreated (29.1 ± 0.6 %) and OJ (27.7 ± 0.4 %)-treated conditions.

OJ and DFOM treatment resulted in a marked increase in apoptosis (8.2 ± 0.9 % (p = 0.015) and 9.1 ± 0.1 % (p = 0.001)) and necrosis (2.8 ± 0.6 % (p = 0.038) and 2.5 ± 0.1 % (p < 0.001)) in SNU-C5/5-FUR cells when compared with untreated cells (2.8 ± 0.1 % for apoptosis and 0.8 ± 0.0 % for necrosis). Co-treatment with OJ and DFOM significantly increase the fraction of apoptosis (14.8 ± 0.7 %, p = 0.002 *vs*. OJ, p = 0.007 *vs*. DFOM) and necrosis (4.0 ± 0.2 %, p = 0.002 *vs*. OJ, p = 0.001 *vs*. DFOM).

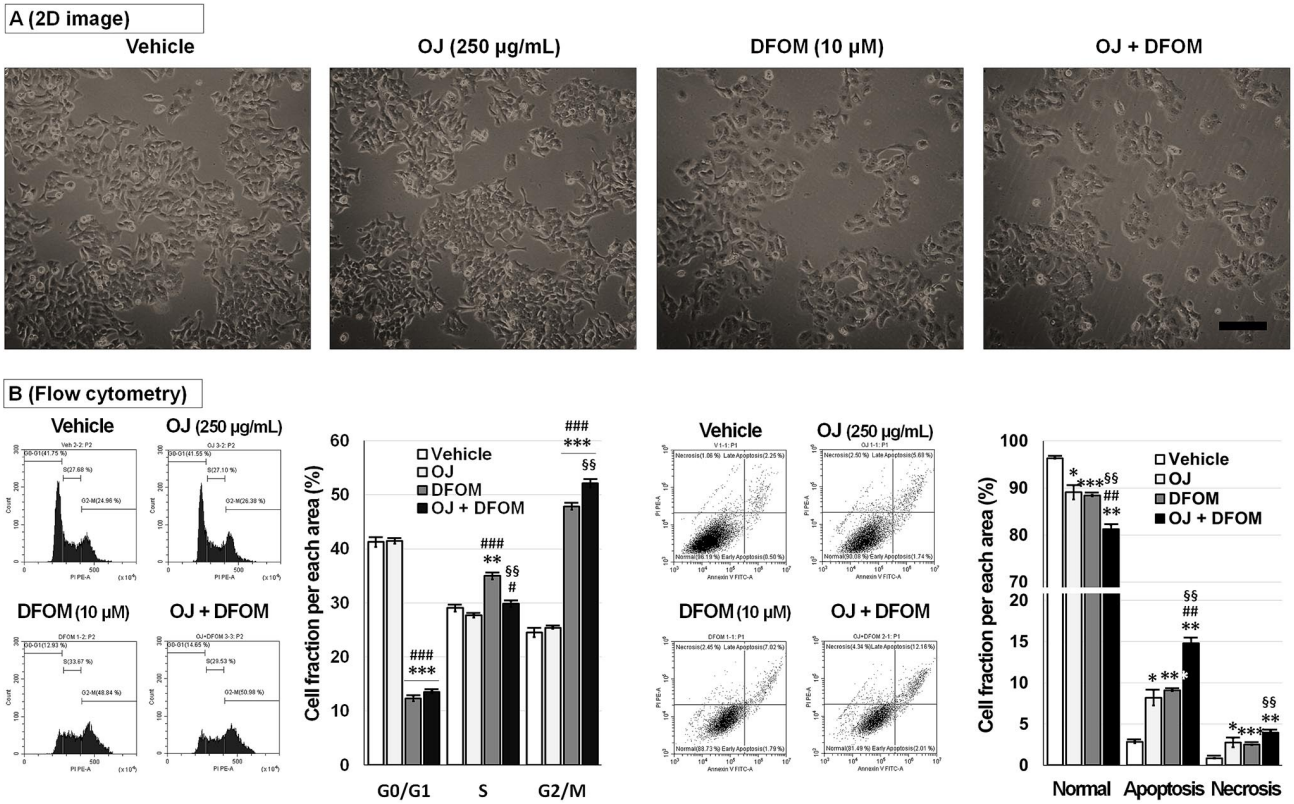

Fig. 4. Co-treatment with deferoxamine mesylate (DFOM) and *Orostachys japonica* (OJ) induces apoptosis as well as G2/M arrest in SNU-C5/5-FUR cells. A. Representative images of SNU-C5/5-FUR cells treated with vehicle or OJ (250 μg/mL), DFOM (10 μM), and OJ + DFOM for 3 days. B. The distribution of cell cycle (left) and cell death (right) in SNU-C5/5-FUR cells were assessed by flow cytometry. DFOM induces cell cycle arrest by increasing G2/M phase, which were further augmented by co-treatment of OJ. OJ and DFOM induces apoptosis, but co-treatment with OJ and DFOM further increased the apoptosis and necrosis fractions with significance. *p < 0.05, **p < 0.01, ***p < 0.001 *vs*. vehicle-treated control; ##p < 0.01, ###p < 0.001 vs. OJ; §§p < 0.01 vs. DFOM.

DISCUSSION

The present study has paid attention to ferroptosis of OJ in SNU-C5/5-FUR CRC cells, since OJ did not induce apoptosis but inhibit proliferation in the previous study (Kim *et al*., 2021). Interestingly, OJ treatment in SNU-C5/5-FUR cells showed different results in animal and cell experiments. Long-term treatment of OJ *in vivo* showed a significant GPX4-dependent HMGB1 increase and an increase in free iron with decreased extracellular transport by FPN. As a result, OJ induces ferroptosis in SNU-C5/5-FUR cells-xenografted tissues. But short-term treatment of OJ *in vitro* induces upregulation of HMGB1, a release marker of ferroptotic cells, despite the transient increase of GPX4, xCT, TfRC and FPN. In ferroptotic CRC, GPX4 and xCT were down-regulated and HMGB1 were upregulated (Chen *et al*., 2020; Park *et al*., 2022). But upregulation of GPX4 (Chen *et al*., 2023a; Zhang *et al*., 2023) and xCT (Luo *et al*., 2023) was also reported in CRC, which could prevent ferroptosis. It can be interpreted that the survived CRC cells when treated with anti-CRC drugs showed the highly expression of GPX4 and xCT including this study. The upregulated GPX4 and

xCT might be linked to greater resistance against to other drugs, and thus inhibiting their activities with natural products, including OJ, might be considered as a new therapeutic strategy. Iron metabolism has somewhat complicated results. FTH1 and FPN were downregulated in KRAS-mutant CRC cells (Chen *et al*., 2020), but upregulated in fenbendazole-treated SNU-C5/5-FUR cells (Park *et al*., 2022).

The potential utility of natural GPX4 and xCT inhibitors in CRC was summarized recently (Zhang & Xie, 2024). The effects of ferroptosis inducers, GPX4 inhibitor and xCT inhibitor, were compared when co-treated with OJ in SNU-C5/5-FUR cells. GPX4 inhibitor did not show any additive effects, but xCT inhibitor showed further inhibition on cell survival. As OJ did not affect on xCT *in vivo* and *in vitro*, the ferroptotic effects of OJ might be depend on GPX4 and could be further augmented with xCT inhibitors including SSZ. In addition, the effects of iron chelator and ferroptosis inhibitor were compared when co-treated with OJ. The inhibitors did not show any significant changes on the cell viability when co-treated with OJ, but high dose of DFOM slightly decreased the cell survival. The decrease in cell viability with OJ and DFOM co-treatment was due to increased apoptosis and cell cycle arrest at G2/M phase. Iron chelation by DFOM might induce cell cycle arrest and ferroptosis-augmented apoptosis as previously suggested (Park *et al*., 2022).

Natural product-mediated chemosensitization based on conventional 5-FU-based chemotherapeutic regimen has been presented as a new therapeutic strategy through activation of ferroptosis in CRC (Sharma *et al*., 2020; Zou *et al*., 2024). A number of natural products showed anti-tumor activity of ferroptosis against CRC cells through downregulation of GPX4 (Chen *et al*., 2020; Zhou *et al*., 2023; Zou *et al*., 2024), which was also observed *in vivo* treated with OJ in this study. The underlying mechanisms of natural products-induced ferroptosis are related to the mitogen activated protein kinase (MAPK) signaling pathways, inhibiting MAPK (Ocampo *et al*., 2020) or extracellular signal-regulated kinase (ERK) (Zou *et al*., 2024). MAPK pathways were also received attention in terms of anti-cancer activity of OJ. While OJ usually activated p38 and ERK (Ryu *et al*., 2014; Kim *et al*., 2019; Kwon *et al*., 2019), OJ extract activated p38 but inhibited ERK (Lee *et al*., 2018) and thus the underlying mechanism of anti-cancer activity was suggested as the p53-dependent cell cycle arrest. Meanwhile, the activation of p38 and ERK was inhibited by methanol extract of OJ (Kim *et al*., 2015), but enhanced by flavonoids from OJ (Lee *et al*., 2015) in leukemia cells. The aqueous extract of OJ, also used in this study, activated p38 (Shim *et al*., 2015; Kim *et al*., 2021) or modulate the activity of ERK (Kim *et al*., 2011, 2021).

Gallic acid can bind to ferroptosis-related genes or targets and regulate the expression of corresponding proteins. As a result, gallic acid might improve overall survival in patient with CRC by regulating ferroptosis (Hong *et al*., 2021). The aqueous extract of OJ, used in this study also, includes gallic acid as an active ingredient (Shim *et al*., 2015; Kim *et al*., 2021). The mechanism of gallic acid was different from OJ extract itself in SNU-C5/5-FUR cells (Kim *et al*., 2021), and thus more detailed further studies should be performed in terms of ferroptosis. In addition to the ferroptosis, natural products showed anti-cancer effects by suppression of β-catenin (Sharma *et al*., 2020). OJ also inhibited the proliferation of MDA-MB-231 breast cancer (Kwon *et al*., 2019) and SNU-C5/5-FUR CRC cells (Kim *et al*., 2021) by suppressing the β-catenin and/or GSK3β expression. Therefore, the relationship between ferroptosis and β-catenin/Wnt signaling pathways should be further investigated in CRC.

Taken together, OJ induces ferroptosis by inhibiting GPX4, TfRC, and FPN, and activating HMGB1 in SNU-C5/5-FUR cells-xenografted tissues although similar changes were not observed *in vitro*. When co-treated with DFOM, OJ further induced apoptosis and cell cycle arrest at G2/M phase. These results suggest that OJ-induced ferroptosis might be related with iron metabolism as well as GPX4. ferroptosis *in vivo* indicating that other pathways might be a feasible candidate to induce ferroptosis or ferroptosis-augmented cell death in 5-FU resistant CRCs. Auriculasin can promote ferroptosis by generating reactive oxygen species, which was blocked by N-acetyl-l-cysteine treatment (Wang *et al*., 2022). Therefore, further detailed ferroptotic pathways related to reactive oxygen species, such as nuclear receptor coactivator 4 (NCOA4), polyunsaturated fatty acid (PUFA), kelch like ECH associated protein 1 (KEAP1), NF-E2-realted factor 2 (Nrf2), and lipoxygenases (LOX), should be investigated to overcome resistance and improve treatment outcomes in CRC.

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YOON, S.P. *Orostachys japonica* induce ferroptosis en células de cáncer colorrectal SNU-C5 resistentes al 5-fluorouracilo. *Int. J. Morphol., 42(6)*:1508-1516, 2024.

RESUMEN: La resistencia a los fármacos es aún una necesidad deficiente en el tratamiento del cáncer colorrectal (CCR). Recientemente, la muerte celular regulada dependiente del hierro, la ferroptosis, ha surgido como una posible estrategia terapéutica para superar la resistencia a los fármacos en el CCR. Como los productos naturales se han probado como candidatos prometedores para inducir ferroptosis en el CCR, se investigaron los efectos anticancerígenos del extracto acuoso de *Orostachys japonica* (OJ)

en células de CCR SNU-C5 (SNU-C5/5-FUR) resistentes al 5-fluorouracilo (5-FU) en términos de ferroptosis. Para el análisis se utilizaron la viabilidad celular, la inmunotransferencia y la citometría de flujo. El OJ mostró una CI50 de 280 µg/mL *in vitro*. El tratamiento a largo plazo con OJ (250 µg/kg) indujo eficazmente la ferroptosis con una disminución de GPX4, receptor de transferrina y ferroportina, y un aumento de HMGB1 en tejidos xenoinjertados con células SNU-C5/5-FUR. El tratamiento a corto plazo con OJ (hasta 250 µg/mL) no mostró una ferroptosis eficaz con un ligero aumento de GPX4, receptor de transferrina y cadena pesada de ferritina. El co-tratamiento con inductores de ferroptosis (RSL3 y sulfasalazina) o inhibidores (mesilato de deferoxamina y ferrostatina-1) no mostró ningún efecto aditivo o sinérgico sobre la viabilidad celular de las células SNU-C5/5-FUR. Cuando se cotrató con mesilato de deferoxamina en dosis altas, el OJ indujo aún más la apoptosis, así como la detención del ciclo celular en la fase G2/M. Los resultados sobre ferroptosis *in vitro* e *in vivo* fueron inconsistentes en las células SNU-C5/5-FUR, pero la ferroptosis *in vivo* indica que otras vías podrían ser viables para inducir ferroptosis o muerte celular aumentada por ferroptosis en los CRC resistentes al 5-FU.

PALABRAS CLAVE: Ferroptosis; *Orostachys japonica*; Resistencia a fármacos; Cáncer colorrectal.

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