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Wharton's Jelly Mesenchymal Stem Cells Derived Secretome Inhibits Colorectal Cancer Cell Growth Via Suppressing Mitophagy

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HIGHLIGHTS

- WJSC-S suppresses mitophagy in colon cancer cells
- WJSC-S suppresses autophagy in colon cancer cells
- WJSC-S reduces ΔΨm and survival of the colon cancer cells
- WJSC-S increases ROS level and apoptosis index in colon cancer cells

Abstract: The anti-cancer effects of Wharton's jelly mesenchymal stem cells (WJSC)-derived secretome (WJSC-S) have been demonstrated in previous studies. However, the anti-cancer mechanism of WJSC-S in tumor suppression remains largely elusive. This study investigated the cytotoxic impacts of WJSC-S by evaluating autophagy and mitophagy on a colon carcinoma cell line (HT-29 cells). The HT-29 cells were treated with 100 µg/mL WJSC-S with or without mitochondrial division inhibitor-1 (Mdivi-1) for 24 hours. MTT test and DAPI staining had used to determine the impacts of WJSC-S on the viability and apoptosis rates of the cells. Mitochondrial membrane potential **(**ΔΨm), reactive oxygen species (ROS) formation, and expression of autophagy and mitophagy-related genes (Parkin and PINK1) had evaluated. Protein levels of the Parkin, PINK1, and LC3-II/LC3-I ratio were also evaluated. WJSC-S reduced ΔΨm and survival of the cells while ROS level and apoptosis index significantly increased. WJSC-S raised the expression of Parkin and reduced PINK1 expression in the HT-29 cells. WJSC-S could also decrease the expression of Beclin-1, ATG5, and ATG12 genes and reduce the LC3-II/LC3-I ratio. Mdivi-1 could enhance WJSC-S impacts on the

cancer cells. These findings show that WJSC-S prevents the proliferation of the HT-29 cells and elevates HT-29 cell death by suppressing the mitophagy process.

Keywords: secretome; mesenchymal stem cells; colon cancer; autophagy; mitophagy.

INTRODUCTION

Colorectal cancer is responsible for about 900,000 death every year [1]. The rate of colon cancer in patients under 50 years has enhanced in recent decades. Despite conventional treatments, the mortality of this cancer is high due to metastasis and drug resistance [2]. Therefore, exploring the new anticancer drugs and their molecular mechanism can help to treat this cancer.

Previous researchers have shown the anti-cancer impact of mesenchymal stem cells (MSCs) on various malignancies [3-5]. Wharton's jelly-MSC (WJSC) inhibits the progression of different cancers in humans and rodents, such as breast, lung, and pancreatic cancer cells [6-8]. MSCs secrete soluble factors (secretome), with various paracrine functions, into the microenvironments [9, 10]. Secretome-derived WJSC (WJSC-S) prevents the progression of different cancerous cells [11-13]. Kalamegam and coauthors have reported that WJSC extracts reduced the level of growth factors, chemokines, and oncogenic cytokines, which inhibit the proliferation of ovarian cancer cells [14].

Mitochondria is a source of energy production and reactive oxygen species (ROS) generation and regulates different types of cell death [15]. ROS, produced by damaged mitochondria, causes DNA damage and contributes to cancer progression. Mitophagy, a specific type of autophagy, promotes mitochondrial removal and prevents the accumulation of damaged mitochondria [16].

According to previous studies, mitophagy enhances resistance to chemotherapy drugs by removing dysfunctional mitochondria [17]. Increasing mitophagy in cancer cells promotes their survival and adaptation to the environment [18, 19] and protects the cancer cells from apoptosis [20]. Following the disruption of mitochondrial membrane potential (ΔΨm), PTEN-induced putative kinase 1 (PINK1) and Parkin proteins establish a large complex to induce mitophagy [15, 16]. PINK1/Parkin is the main pathway of mitophagy [21].

The high expression of PINK1 protects cells against various cytotoxic agents and increases the survival of tumor cells [22]. Beclin-1 regulates Parkin transport to the mitochondria before autophagosome formation [23]. High expression of the ATG5, ATG12, and LC3-II (autophagy-related proteins) stimulates mitophagy [24].

In this study, the effect of human WJSC-derived secretome (WJSC-S) on mitophagy in the HT29, a colon cancer cell line, has been investigated.

MATERIAL AND METHODS

Secretome preparation

The WJSCs were purchased from Royan Institute, Tehran, Iran. The WJSCs were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS) and 1% Pen/Strep. At 80% confluency, the cells were washed and incubated in serum-free media overnight. The removed conditioned media had centrifuged at 5,000.g for 10 min. Then, the conditioned medium was re-centrifuged at 5,000.g with an Amicon Ultra-15 centrifugal filter. The protein concentration was determined by a BCA kit (Invitrogen) and maintained at -80 °C.

Cell culture

HT-29 cells, after purchasing from Pasture Institute (Iran), were cultured in McCoy's 5a medium containing two mM glutamine and FBS (10%) and incubated in a moist condition with 5% CO2 at 37˚C. At 80% confluency, the HT-29 cells had treated with 100 µg/mL WJSC-S for 24 hours. Effective concentration and duration time of WJSC-S were based on the MTT assay (Table 1). HT-29 cells were exposed to Mdivi-1 (10 µM; Sigma) and WJSC-S (100 µg/mL) for 24 hours.

Table 1. The IC_{50} (%) of different concentration of WJSC-S on the viability of HT-29 cells.

Treatment	12 hours	24 hours	48 hours
25 µg/mL	98.6 ± 1.4	97.4 ± 1.8	98.1 ± 1.9
50 µg/mL	96.3 ± 3.9	74.6 ± 4.9*	71.3 ± 5.1*
100 µg/mL	95.3 ± 3.3	48.8 ± 2.6**	32.6 ± 3.7**

Values are expressed as mean ± SD (n=5). * $p < 0.05$, ** $p < 0.05$; * symbol indicate comparison to 12 hours.

Cell viability

At first, the cells were cultured in 96-well dishes (10^4 cells/ well) for 24 hours. The cells were exposed to different concentrations of WJSC-S at different times. Then, 0.5 mg/mL MTT solution was poured into the well and incubated for three hours at 37°C. When the supernatant was deleted, DMSO (100 µL) was poured into the wells. After 30 min, the absorbance was recorded at 570 nm using a BioRad microplate reader.

DAPI staining

The HT-29 cells were cultured in 6-well plats (500 cells per well) and treated with Mdivi-1 or WJSC-S according to the four groups. The cells were incubated in 4 % paraformaldehyde for ten min at room temperature. After washing, DAPI (4′, 6-diamidino-2-phenylindole; 2 mg/ mL in PBS) was added and maintained for 10 min. In this method, apoptotic cells have condensed chromatin and show a highly bright nucleus under a fluorescence microscope (Olympus, Japan). As previously described, the number of highly bright nuclei was divided by the total number of nuclei and multiplied by 100 to calculate the apoptotic index [25].

Real-time PCR

The RNA of the HT-29 cells (1,000,000 cells) was extracted by an RNeasy kit (Takara). A cDNA kit (Qiagen) was applied to generate cDNA from the isolated RNAs. The cDNAs were amplified in the PCR reaction buffer containing SYBR Green, and primers (Table 2). A 45-cycle program was used for PCR amplification: initial denaturation (95ºC, 10 seconds); denaturation (995ºC, 15 seconds); annealing (55ºC, 20 seconds); and extension (60ºC, 20 seconds). The housekeeping GAPDH gene was applied to normalize the relative gene expression. The data were analyzed using the $2^{-\Delta\Delta CT}$ formula.

Table 2. Primer sequences

Genes	Forward	Reverse
Parkin	CTGGAAGTCCAGCAGGTAGAT	TCATCCCAGCAAGATGGACC
PINK1	CAAGAGAGGTCCCAAGCAAC	GGCAGCACATCAGGGTAGTC
Bax	GGATGCCTTTGTGGAACTGT	TCACTTGTGGCCCAGATAGG
Bcl-2	ACCCAGAAGACTGTGGATGG	TTCTAGACGGCAGGTCAGGT
Beclin-1	CGGTTTTTCTGGGACAACAA	AAAAACGTGTCTCGCCTTTC
ATG5	CCAGAAAAAGACCTTCTGCACT	CAATCCCATCCAGAGTTGCT
ATG12	AAGATGGCAGAAGACCCAGA	TGAAGTCGATGAGTGCTTG
GAPDH	ACCCAGAAGACTGTGGATGG	TTCTAGACGGCAGGTCAGGT

ELISA method for detecting protein levels

ELISA assay kits had used to determine the protein level of PINK1, Parkin, and LC3-II/LC3-I ratio according to the manufacturer's instructions. In short, the treated cells were washed and mixed with radioimmunoprecipitation (RIPA) lysis buffer with protease inhibitor. BCA assay kit (Sigma, USA) was used to determine the protein amount of the cells or isolated mitochondria. The proteins were bound to the primary antibodies and detected by a horseradish peroxidase-conjugated secondary antibody. Quantification was done by recording the optical density at 450 nm.

Detecting ROS level and ΔΨm

A Mitochondria Isolation Kit (Sigma) had used to isolate mitochondria from the H-29 cells. The protein concentration of the isolated mitochondrial suspension was quantified by a BCA kit. The amount of 0.5 mg protein/ mL was mixed with 10 µM Rhodamine-123 for ten min. The fluorescence was detected by a spectrophotometer (emission: 535 nm; excitation: 490 nm). The ROS level was measured by a dichlorofluorescein-diacetate assay kit based on the Company's guides. The ROS level of the treated cells (10^5) was measured in Em: 570 nm and Ex: 490 nm.

Statistical Analysis

Statistical analyses had done with the SPSS 21.0 software. A one-way analysis of variance followed by Tukey's test had used for analyzing the data. The Kruskal-Wallis test had used for nonparametric data. P-value < 0.05 was significant.

RESULTS

Viability

WJSC-S decreased the survival of the colon cancer cells compared to the control ($p < 0.01$).

The survival rate (Figure 1) of the Mdivi-1-exposed cells was significantly decreased compared to the control ($p < 0.01$).

WJSC-S in combination with Mdivi-1 decreased the survival of the cells compared to the WJSC-S ($p < 0.05$), Mdivi-1 ($p < 0.05$), and control ($p < 0.001$) groups (Figure 1).

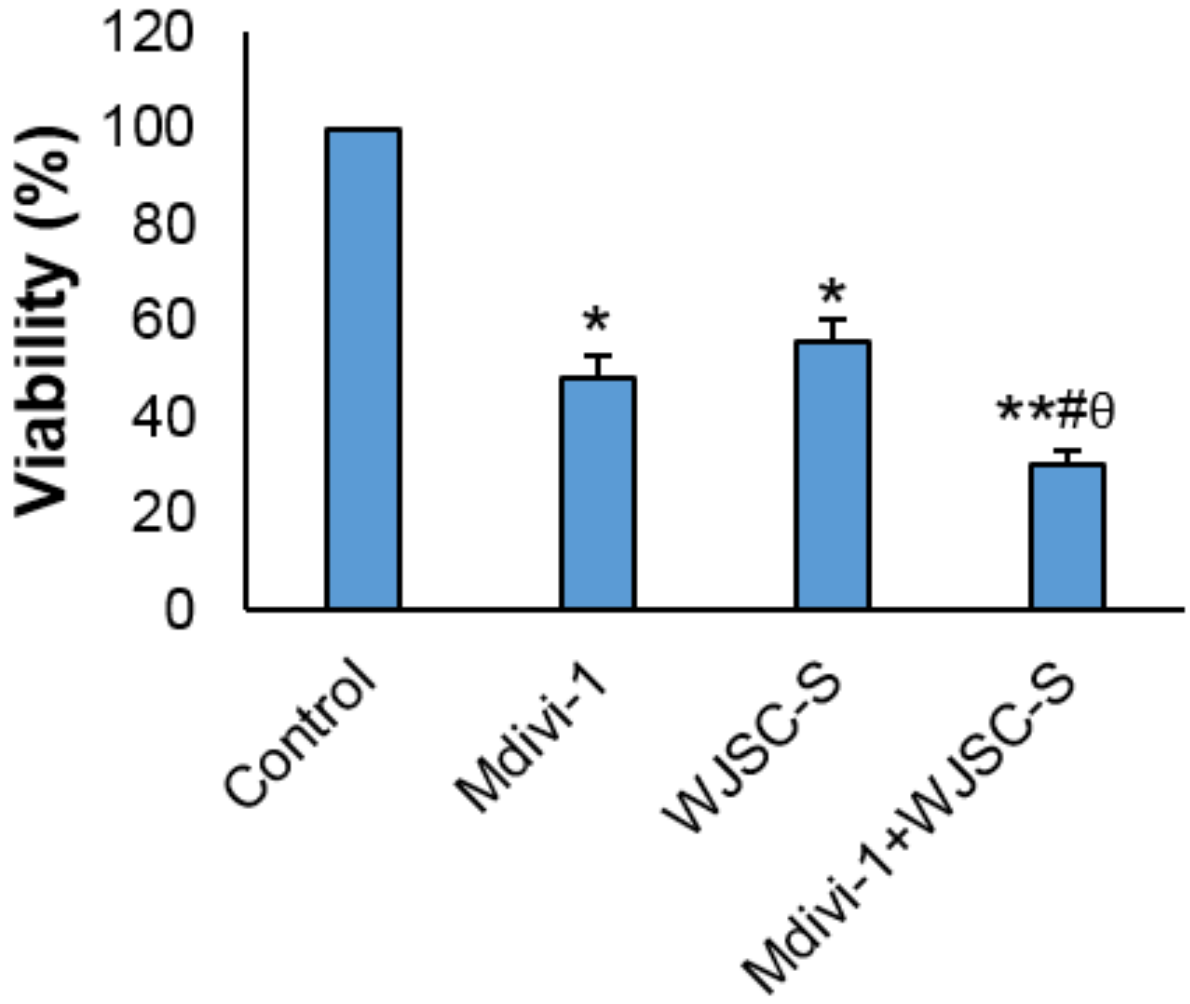

Figure 1. Viability of the HT-29 cells in various groups (mean ± SD; n=6). * $p < 0.01$, ** $p < 0.001$, #$p < 0.05$, θ $p < 0.05$; * , #, and θ show comparison with the control, WJSC-S, and Mdivi-1 groups.

Apoptosis assessment

As reported in Figure 2, a high population of normal nuclei was observed in the control group. The percentage of apoptotic cells in the Mdivi-1 ($p < 0.001$) and WJSC-S ($p < 0.01$) groups significantly increased. In the WJSC-S group, the apoptotic index was significantly lower than in the Mdivi-1 group ($p < 0.01$). Co-treatment Mdivi-1 with WJSC-S enhanced apoptosis percentage compared to the WJSC-S ($p < 0.01$) and Mdivi-1 ($p < 0.05$) groups.

As displayed in Figure 2, Mdivi-1 and WJSC-S alone enhanced the Bax/Bcl-2 ratio ($p < 0.01$ and $p < 0.05$, respectively). In the WJSC-S group, the Bax/Bcl-2 ratio was significantly lower than in the Mdivi-1 group ($p < 0.05$). Co-treatment Mdivi-1 with WJSC-S increased the Bax/Bcl-2 ratio compared to the control ($p < 0.001$), WJSC-S ($p < 0.01$) and Mdivi-1 ($p < 0.05$) groups.

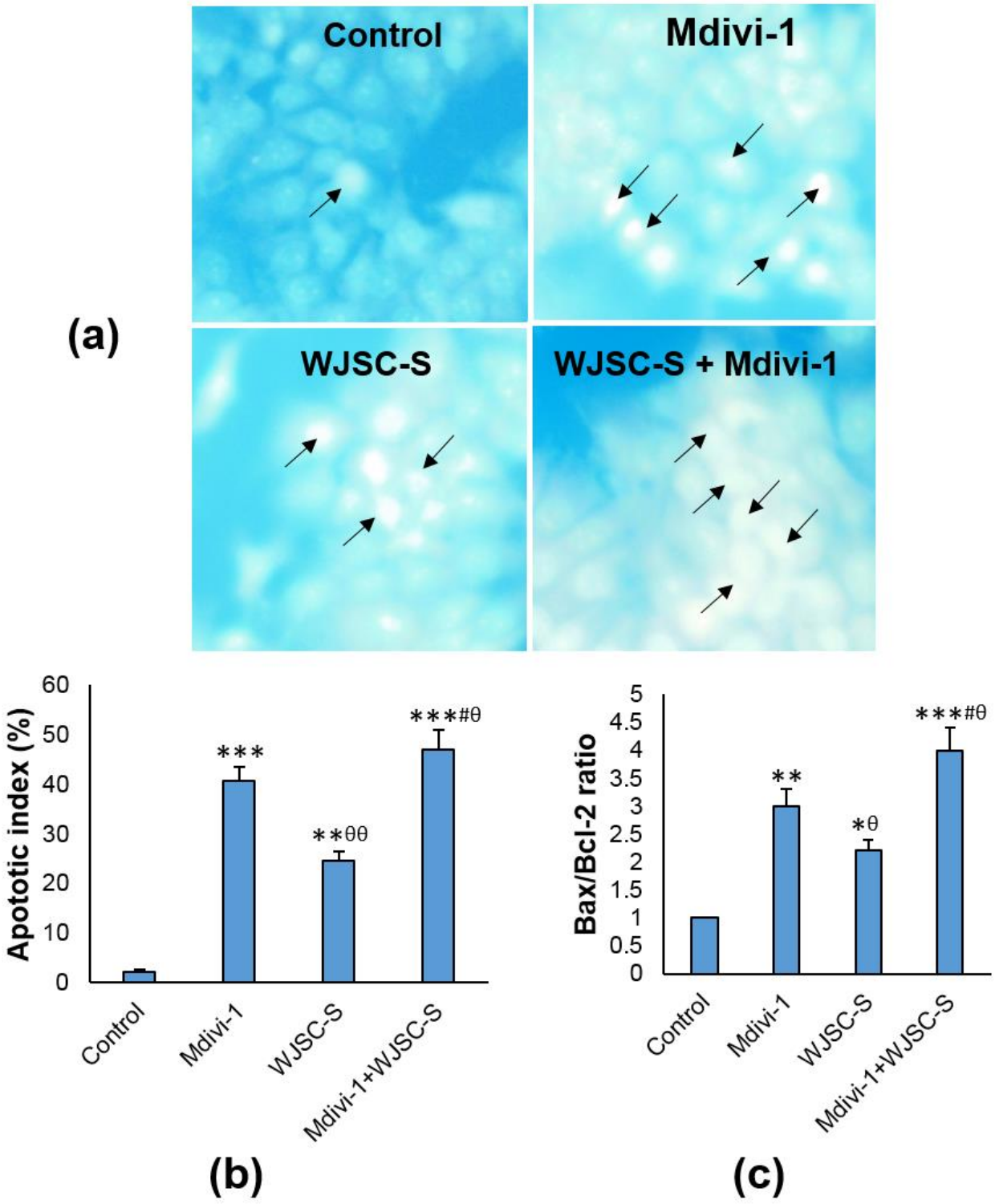

Figure 2. (a) DAPI staining (arrows indicate apoptotic cells), (b) apoptotic index and (c) Bax/Bcl-2 ratio (RT-PCR results) in different groups (mean ± SD). * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, # $p < 0.01$, θ $p < 0.05$, θθ $p < 0.01$; * , #, and θ show comparison with the control, WJSC-S, and Mdivi-1 groups.

Protein level and mRNA expression of autophagy and mitophagy-related genes

WJSC-S significantly diminished mRNA expression of Beclin-1, ATG-5, and ATG-12 compared with the control ($p < 0.01$). WJSC-S significantly reduced the mRNA expression of PINK1 in the HT-29 cells ($p < 0.001$). WJSC-S significantly increased the Expression of the Parkin gene ($p < 0.001$) (Figure 3).

In the WJSC-S, the LC3-II/LC3-I ratio was significantly diminished compared to the control ($p < 0.001$). WJSC-S significantly reduced the protein level of PINK1 in the HT-29 cells ($p < 0.01$). Expression of the Parkin protein significantly increased ($p < 0.05$) (Figure 4).

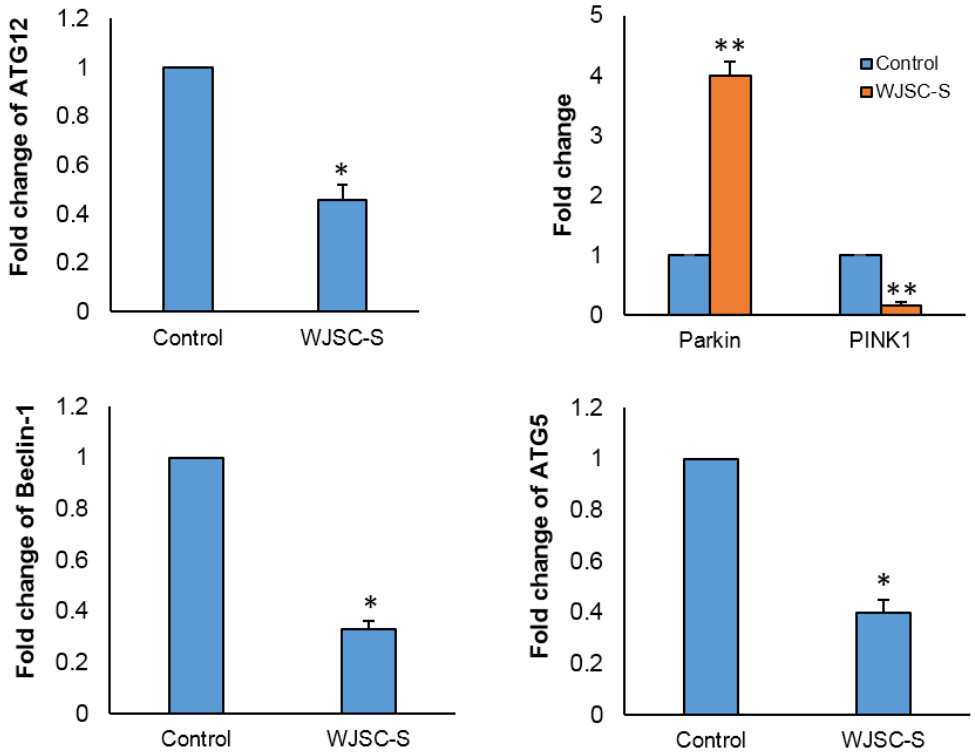

Figure 3. mRNA expression (fold change) of autophagy and mitophagy-related genes (mean ± SD). * $p < 0.01$, ** $p < 0.001$

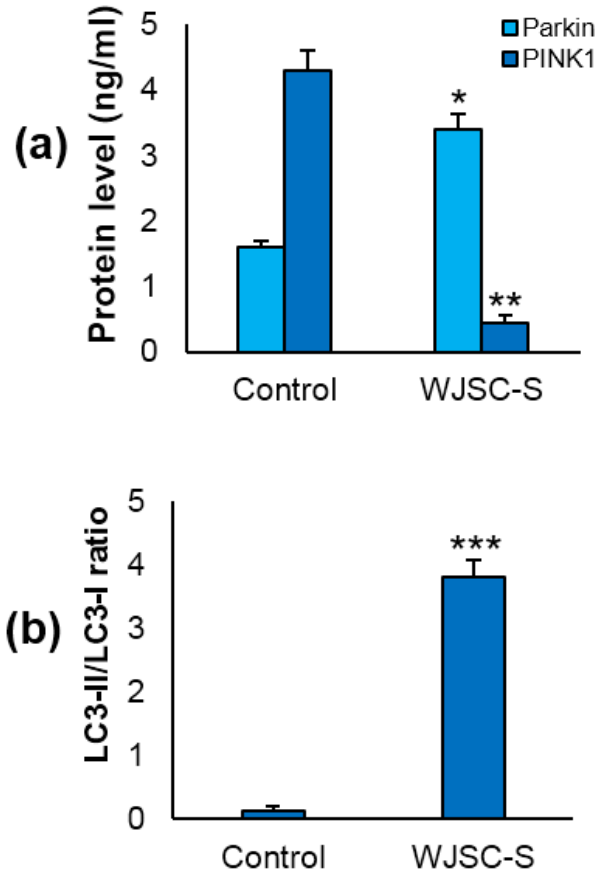

Figure 4. Protein expression (a) and LC3II/LC3I ratio (b) of different groups (mean ± SD). * $p < 0.01$, ** $p < 0.01$, *** $p < 0.001$

ΔΨm and ROS level

ΔΨm decreased in the WJSC-S and Mdivi-1 groups ($p < 0.01$). The ΔΨm of the WJSC-S+ Mdivi-1 groups significantly diminished compared to the control ($p < 0.01$). Mdivi-1-exposed cells showed no significant change in the ROS level compared to the control (Figure 5).

WJSC-S significantly enhanced ROS level compared to the Mdivi-1 ($p < 0.001$) and control ($p < 0.001$) groups. WJSC-S + Mdivi-1 significantly elevated ROS generation compared to the control ($p < 0.001$), Mdivi-1 ($p < 0.001$), and WJSC-S ($p < 0.05$) groups (Figure 5).

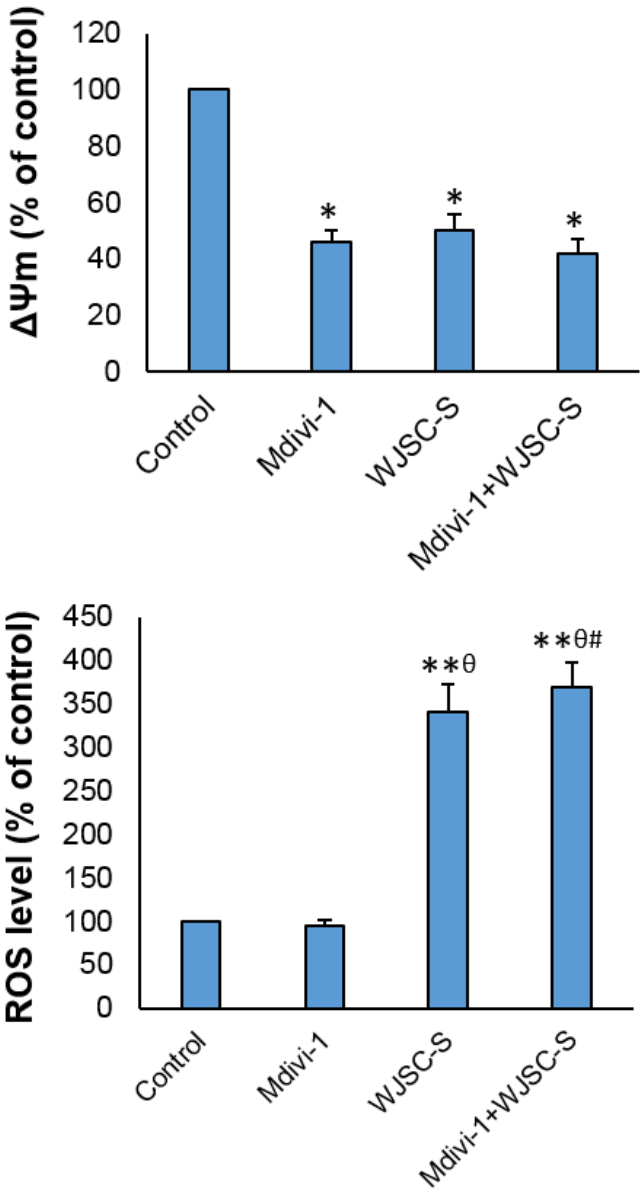

Figure 5. ΔΨm and ROS levels in different groups (mean ± SD). * $p < 0.01$, ** $p < 0.001$, # $p < 0.05$, θ $p < 0.001$. *, #, and θ show comparison with the control, WJSC-S, and Mdivi-1 groups.

DISCUSSION

The effect of WJSC-S on the HT-29 cells was explored by evaluating the mitophagy process in this work. The WJSC-S could reduce the viability of the HT-29 cells. In parallel with our finding, Rezaei and coauthors (2020) showed that WJSC-S inhibits the proliferation of the HT-29 cells [26]. Mirabdollahi and coauthors (2020) have reported that the WJSC-S significantly inhibits the growth of the breast cancer cell line (MCF-7 and 4T1) in vitro and also increases the survival of the mouse model of breast cancer [27]. WJSC-derived microvesicles reduced the proliferation of bladder cancer cells in mice [28]. In contrast, De Castro and coauthors (2017) found that two glioblastoma cell lines, U251 and SNB-19, increased their survival, proliferation, and invasive strength when exposed to WJSC-S [29].

Interestingly, the growth and proliferation of U87MG glioblastoma cells decreased significantly in the face of WJSC-S [30]. Thus, the secretome plays a dual role in the regulatory mechanisms of cancer cells and can either inhibit or promote the growth of tumor cells [31, 32].

The decreasing survival of the HT-29 cells was accompanied by increasing apoptosis in the WJSC-S group (Figures 1 and 2). In line with these results, Rezaei and coauthors (2020) showed that WJSC-S increases apoptosis in the HT-29 cells by activating the intrinsic (mitochondrial-dependent) apoptosis pathway [26]. In our study, WJSC-S significantly decreased ΔΨm, which indicated mitochondrial-dependent apoptosis (Fig. 5).

The decreasing ΔΨm accompanied by the enhancing ROS generation in the WJSC-S group. ROS generation regulates the autophagy process, which promotes the degradation of damaged mitochondria [33]. Previous studies have reported that anti-cancer drugs inhibit cancer cell survival by suppressing autophagy [34, 35]. The decreased LC3-II protein leads to the suppression of mitophagy and subsequently increases ROS generation [36]. WJSC-S suppressed autophagy by reducing the LC3-II/LC3-I ratio and down-regulation expression of Beclin-1, ATG5, and ATG12 genes (Figures 3 and 4).

To evaluate the role of mitophagy on the decreased survival of the HT-29 cells, we examined the expression of mitophagy-related genes. In this study, WJSC-S could significantly reduce PINK1 expression. High expression of PINK1 enhances resistance to chemotherapy and oxidative stress in prostate cancer cells [37].

Another study showed that the expression of PINK1 may be associated with tumorigenesis and the progression of lung cancer [38].

The expression of Parkin increased by WJSC-S in the cancerous cells (Figures 3 and 4). Parkin suppression relates to the progression of breast, ovary, and lung tumors [39-41]. Fujiwara and coauthors found that the proliferation of hepatocellular carcinoma cells increases in mice with the Parkin gene turned off [42]. Parkin downregulation increases susceptibility to tumorigenesis, indicating a tumor-suppressive function for Parkin [16].

To confirm the role of mitophagy on the decreased survival of the HT-29 cells, we used Mdivi-1 as a mitophagy inhibitor. Indeed, Mdivi-1 could decrease the survival rate and raise the apoptosis index and Bax/Bcl-2 ratio in the HT-29 cells. Mdivi-1 decreased the proliferation of lung and colon cancer cell lines (H460, A549, and HCT116) via increasing apoptosis [43]. Chemotherapy drugs such as Doxorubicin, Salinomycin, and UNBS1450 are more effective when mitophagy is inhibited [44].

According to our results, Mdivi-1 and WJSC-S may have synergistic or additive effects and enhance apoptosis in the HT-29 cells. Tang and coauthors found that PINK1/ Parkin knockdown or mitophagy inhibitors enhance magnolol-induced cell death both in vitro and in vivo [45].

The co-treatment of Mdivi-1 with WJSC-S enhanced oxidative stress with an increase in ROS generation and reduced ΔΨm, which is in line with previous studies [46].

CONCLUSION

The present study revealed the cytotoxic impacts of WJSC-S on the HT-29 cells. Since the inhibition of mitophagy by Mdivi-1 promoted WJSC-S-induced cell death and reinforced WJSC-S's anticancer efficacy, we concluded that the preventive impact of WJSC-S against HT-29 cell growth is mediated by the suppression of mitophagy signaling pathway.

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Conflict of interest: The authors declared no conflict of interest.

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