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Original Article

The effects of chitosan-loaded JQ1 nanoparticles on OVCAR-3 cell cycle and apoptosis-related gene expression

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Abstract

Background and purpose: Ovarian cancer is the deadliest gynecological cancer. Bromodomain and extra terminal domain (BET) proteins play major roles in the regulation of gene expression at the epigenetic level. Jun Qi (JQ1) is a potent inhibitor of BET proteins. Regarding the short half-life and poor pharmacokinetic profile, JQ1 was loaded into newly developed nano-carriers. Chitosan nanoparticles are one of the best and potential polymers in cancer treatment. The present study aimed to build chitosan-JQ1 nanoparticles (Ch-J-NPs), treat OVCAR-3 cells with Ch-J-NPs, and evaluate the effects of these nanoparticles on cell cycle and apoptosis-associated genes.

Experimental approach: Ch-J-NPs were synthesized and characterized. The size and morphology of Ch-J-NPs were defined by DLS and FE-SEM techniques. OVCAR-3 cells were cultured and treated with Ch-J-NPs. Then, IC_{50} was measured using MTT assay. The groups were defined and cells were treated with IC_{50} concentration of Ch-J-NPs, for 48 h. Finally, cells in different groups were assessed for the expression of genes of interest using quantitative RT-PCR.

Findings/Results: IC_{50} values for Ch-J-NPs were 5.625 µg/mL. RT-PCR results demonstrated that the expression of genes associated with cell cycle activity (c-MYC, hTERT, CDK1, CDK4, and CDK6) was significantly decreased following treatment of cancer cells with Ch-J-NPs. Conversely, the expression of caspase-3, and caspase-9 significantly increased. BAX (pro-apoptotic) to BCL2 (anti-apoptotic) expression ratio, also increased significantly after treatment of cells with Ch-J-NPs.

Conclusion and implications: Ch-J-NPs showed significant anti-cell cyclic and apoptotic effects on OVCAR-3 cells.

Keywords: Chitosan; JQ1; Nanoparticles; Ovarian cancer.

INTRODUCTION

Ovarian cancer is the deadliest gynecological cancer. Each year, nearly 240,000 new cases of women with ovarian cancer are diagnosed worldwide (1). The type of ovarian cancer in women over 40 age in 90% of cases is epithelial (2). The ovarian cancer 5-year survival rate in a combined all stages, is 49% (3). Despite recent efforts for better management of ovarian cancer (4-8), therapeutic strategies are still limited to surgery and chemotherapy (9). The epigenetic alterations in the expression of tumor suppressor genes, or proto-oncogenes can lead to pathologic conditions including cancer (10). Three main mechanisms involved in these alterations are histone modifications, DNA and RNA methylations, and non-coding RNAs (11). The interaction between histone and DNA weakens by the addition of acetyl groups to lysine residues of histones. This neutralizes the charge on histones, increases chromatin accessibility, and enhances the possibility of transcription (12).

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Bromodomain and extra terminal domain (BET) is a family of proteins of four members (BRD2, BRD3, BRD4, and BRDT) that play major roles in cell cycle regulation (13). These proteins own two tandem bromodomains that recognize and bind to acetylated lysine in histones and transcription factors. BRD4 is the most important member of this family with several roles in transcriptional regulation (14). Inhibitors of BET proteins may function as anticancer drugs at the epigenetic level (15). In 2010, different research groups successfully developed two BET inhibitors, Jun Qi (JQ1) (16) and BET-I (17). It was first developed by Jun Qi at Brigham and Women's Hospital. JQ1 is a specific small molecule inhibitor of BETs, particularly BRD4. It competes with BET bromodomains in binding to acetyl-lysine recognition pockets (16). Since its synthesis, JQ1 has been reported as an active chemical substance against several liquid and solid tumors. JQ1 also has shown promising effects in animal models of diseases and human cancer cell lines, hematopoietic stem cells (18), hepatic stellate cells (19), thyroid cancer cells (20), Huntington's disease (21), and brain plasticity (14,22). In ovarian cancer, it is suggested that JQ1 is associated with cell cycle arrest and induction of apoptosis (23). It has been reported that JQ1 in combination with cisplatin suppressed the JAK-STAT signaling and inhibited the growth of ovarian tumors. Besides, JQ1 enhances the sensitivity of ovarian cancer cells to cisplatin (24). To achieve the most efficacy and safety, drugs are better to be delivered at a particular controlled rate. Hence, controlled delivery systems are developed to overcome the problems associated with conventional drug delivery procedures (25). Regarding the short half-life and the poor pharmacokinetic profile, JQ1 was loaded into newly developed nano-carriers (26). Breakthroughs in producing materials in nanoscales have provided promising tools for the controlled delivery of drugs in cancer and diseases (27,28). Chitosan-based nanoparticles are among those of interest. Chitosan is made by deacetylation of chitin. Chitin, the second most abundant natural polysaccharide (after cellulose), is part of the components of crustacean shells. Chitosan is biodegradable and biocompatible and actively is used in biomedical approaches (29-32). Chitosan nanoparticles escape the endosomes, hence, the preset potential for nuclear delivery (3,9,33). It was suggested that chitosan potentially inhibits ovarian cancer proliferation (34). Similar effects have been demonstrated but with more promising effects when chitosan is loaded with other materials to make nanoparticles (35,36). In this study, we aimed to build and characterize chitosan-loaded JQ1 nanoparticles and to investigate the expression of cell cycle-associated genes (c-MYC, human telomerase reverse transcriptase (hTERT), CDK1, CDK4, CDK6), and apoptotic associated genes (caspase 3, caspase 8, caspase 9, BAX, BCL2) after treatment with these nanoparticles. c-MYC is a master regulator of cell proliferation (37) and a key transcription factor in ovarian cancer (24). hTERT is activated in 90% of cancer cells (38). Cyclin-dependent kinases (CDK1, CDK4, and CDK6) play important roles in the control of cell division and transcriptional regulation (39). Caspase 3, an executioner caspase; and caspase 9 and caspase 8, two initiator caspases are associated with tumor invasiveness and aggressiveness (40,41). BCL2 and BAX are two members of this family that show opposite functions in the intrinsic apoptotic pathway. BCL2 is anti-apoptotic and BAX is pre-apoptotic. Hence, the high ratio of Bax / Bcl2 is in favor of apoptosis (42,43).

MATERIAL AND METHODS

Materials

JQ1 was purchased from Tocris Bioscience, Bristol, UK (Cat. No. 4499). Chitosan was purchased from Sigma-Aldrich, Munich, Germany (CAT No. 9012-76-4). Culture media and growth supplements were purchased from Gibco (Grand Island, NY, USA) and Sigma Chemical Co. (St. Louis, MO, USA). OVCAR-3 cells (NCBI code. C430) were supplied by Pasteur Institute, Iran. Normal granulosa cells (GCN-1) were provided by the Iranian Biological Resource Center.

Synthesis of chitosan nanoparticles and loading with JQ1

Nanoparticles were formulated by the ionotropic gelation method (44,45). Briefly, 100 mg of chitosan (deacetylated chitin, poly (D-glucosamine), 375 kDa, viscosity of 200-800 Kcps; 90% deacetylated) dissolved in 50 mL of 5% glacial acetic acid at 40 ℃ by stirring. The stock of 4 µM/mL (+)-JQ-1 was prepared (dimethyl sulfoxide (DMSO) used as solvent). In a dropwise addition method, JQ1 was added to chitosan solution with the proportion of 1/1 and stirred for 60 more min (solution A). Next, 0.1% tripolyphosphate (TPP) in deionized water, was added to solution A and stirred for 30 min. The final solution was centrifuged at 13000 rpm, for 15 min. Finally, chitosan-JQ1 nanoparticles (Ch-J-NPs) were separated from the obtained two-phasic solution (water/nanoparticles).

Characterization of Ch-J-NPS

Particle size measurement by dynamic light scattering (DLS) method

Dynamic light scattering (DLS) method was performed to find the size of nanoparticles in dispersion (SZ100, Horbia, Japan). DLS estimates the size according to the light that is scattered by the particles under Brownian motion. Using this technique, the hydrodynamic diameter, polydispersity index (PDI), and zeta potential of the particles were determined (46, 47).

Field emission scanning electron microscopy

Field emission scanning electron microscopy (FE-SEM; TESCAN, MIRA III, 20 kV, Czech) equipped with energy-dispersive X-ray spectroscopy used for FE-SEM. FE-SEM is preferred over SEM due to the superior quality of images (48). It is used to study surface morphology and structures of Ch-J-NPs. The steps were performed following the previously described protocol (49). Briefly, a monolayer of dry Ch-J-NPs was placed on an aluminum stab using carbon tape. Then, using a gold film, the sample was coated and examined.

Cell culture

OVCAR-3 cells cultured in RPMI-1640 media supplemented with 10% fetal calf serum. Normal granulosa cells (GCN-1) were kindly provided by the Iranian biological resource center, and cultured in DMEM/Ham's F-12 medium supplemented with 10% fetal bovine serum (FBS). Both culture media were also supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 4mM L-Glutamine, and 2 mM non-essential amino acids, and then incubated at 37 °C in 5% CO2 and 95% humid air.

Evaluation of the Ch-J-NPs effect on cell viability

The 50% inhibitory concentration (IC_{50}) is widely used to measure the effectiveness of a given anticancer therapeutic at reducing cancer cell viability *in vitro* (50). To determine the IC_{50} concentration of Ch-J-NPs, cells were seeded on a 96-well plate at a density of 4×10^3 /well and incubated overnight. Then, cells were treated with different concentrations of Ch-J-NPs (1, 5, 10, 20, and 40 µg/mL) for 48 h. Next, to evaluate cell viability, MTT (3-(4,5- dimethylthiazol -2-yl)-2,5- diphenyl-2H-tetrazolium bromide) assay was performed based on the previously described protocol (51). In brief, 5 µL of MTT (5 mg/mL, Sigma, USA) was added to each well and incubated for 1 h. Then, DMSO was added to each well to terminate the MTT reaction. Finally, the absorbance was measured at 570 nm.

Experimental groups

Four groups were defined for this study including tumor group, untreated OVCAR-3 cancer cells; tumor + JQ1, OVCAR-3 cells treated with JQ1 for 48 h; tumor + (Ch-J-NPs) group, OVCAR-3 cells treated with Ch-J-NPs for 48 h; and normal Epi + (Ch-J-NPs), normal granulosa cells (GCN-1) treated with Ch-J-NPs for 48 h.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from cells in different groups using Trizol reagents (#011001 Kiazol, Kiazist, Iran). cDNA was synthesized, using an Easy cDNA synthesis kit (Parstous, Iran) as per the protocol. Quantitative real-time polymerase chain

reaction (qRT-PCR) performed using Add SYBR master (Ref 70201, AddBio, Korea). Data were normalized to the fold chain of GAPDH (reference gene) and relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method. Primers for human genes (c-MYC, hTERT, CDK1, CDK4, CDK6, BCL2, BAX, caspase3, caspase8, caspase9) were synthesized by SinaColon, Iran (Table 1).

Table 1. Primers for human genes.

BCL2	Forward: TGGTCTTCTTTGAGTTCGG
	Reverse: GGCTGTACAGTTCCACAA
BAX	Forward: CGCCCTTTTCTACTTTGACA
	Reverse: GTGACGAGGCTTGAGGAG
Caspase 3	Forward: GGACTGTGGCATTGAGAGAG
	Reverse: GGAGCCATCCTTTGAACTTC
GAPDH	Forward: CTTTGGTATCGTGGAAGGAC
	Reverse: GCAGGGATGATGTTCTGG
CDK1	Forward: CAGAGTAGAAAGTGAAGAGGAAG
	Reverse: GTAGTGACCAGGAGGGATAGA
CDk4	Forward: TGCTGCTGGAAATGCTGACTT
	Reverse: GGTGGGAGGGGAATGTCATTA
CDK6	Forward: TTTGATGTTTGGTTCTGGCGG
	Reverse: TTTTTAAGATTAGGTCCTTTT
CASP8	Forward: CAGCAAAGAGAGAAGCAGCAG
	Reverse: CCCCGAGGTTTGCTTTTCATT
CASP9	Forward: GCGACCTGACTGCCAAGAAAA
	Reverse: GCGACCTGACTGCCAAGAAAA
TERT	Forward: ATGAGTGTGTACGTCGTCGAG
	Reverse: ACCCTCTTCAAGTGCTGTCTG
c-MYC	Forward: GGCGTGGGGGAAAAGAAAAAA
	Reverse: TTCTTTTTCCCGCCAAGCCTC

Statistical analysis

One-way ANOVA followed by a post-hoc Tukey test was used to define significant differences in gene expression between groups. The statistical analysis was done using GraphPad Prism software, 9.5.0. version. Data are expressed as means ± SD. *P*-values ≤ 0.05 were considered statistically significant.

RESULTS

Characterization of Ch-J-NPs

Particle size measurement

The size distribution of Ch-J-NPs was determined by measuring the random changes in intensity when light scattered from the solution. DLS results were analyzed by the cumulants method and calculation of the Z-average. The Zetasizer software reported Ch-J-NPs size distribution by intensity (Fig. 1), volume, and number, as well as size statistics. The results indicated that the average radius of Ch-J-NPs was in the range of 349.1 r.nm. As are found in supplementary data, all three distributions (intensity, volume, and number) have similar Z-average, as well as similar PDI (polydispersity index).

FE-SEM

The high-magnification FE-SEM images of the Ch-J-NPs shown in Fig. 2. A and B demonstrated the superficial morphology of our nanoparticles with a roughly spherical form. It plainly showed the presence of JQ1 nanoparticles on the prepared Ch-J-NPs.

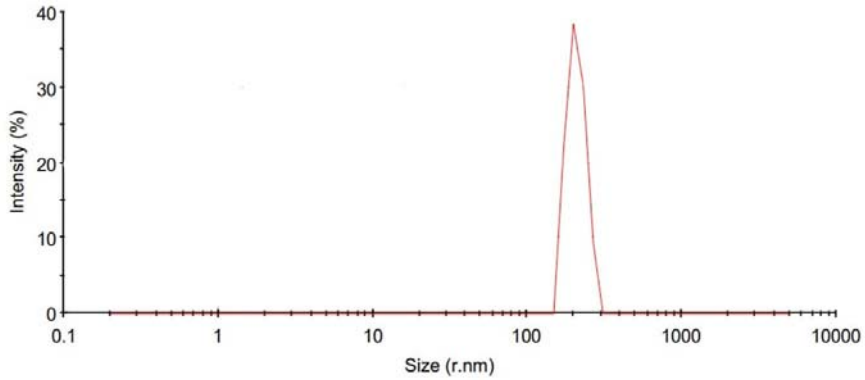

Fig. 1. Size distribution of the chitosan-JQ1 nanoparticles was measured by the Malvern Zetasizer instrument. The particle size (nm) is represented by the horizontal coordinate and the intensity is represented by the vertical coordinate (%). All particle sizes are concentrated in the range of one peak.

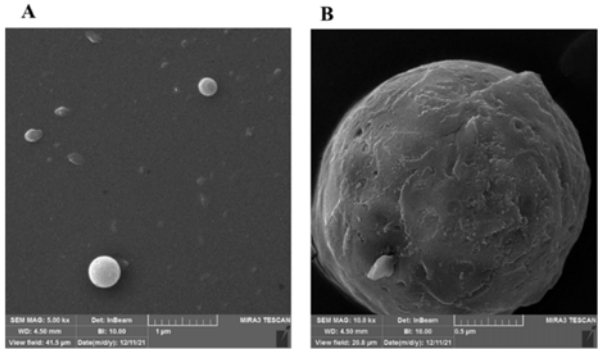

Fig. 2. The high-magnification field emission scanning electron microscopy images of the chitosan-JQ1 nanoparticles with (A) 5 K and (B)10 K magnification.

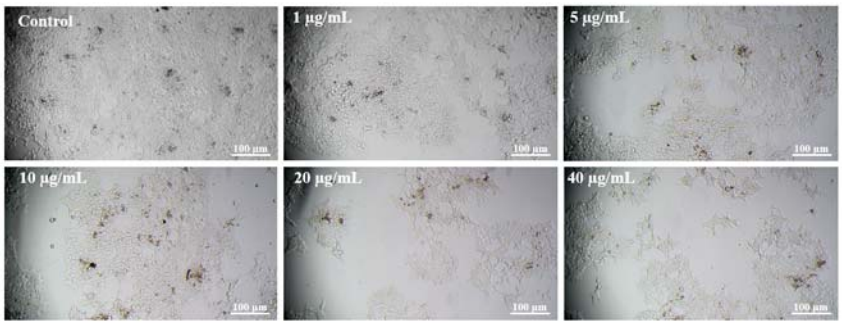

Fig. 3. OVCAR-3 ovarian cancer cells without treatment (control), and after 24-h treatment with different concentrations (0, 1, 5, 10, 20, and 40 µg/mL) of chitosan-JQ1 nanoparticles.

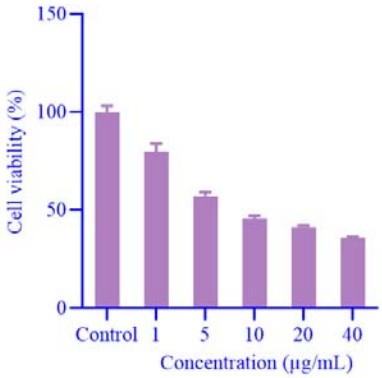

Fig. 4. Cell viability after treatment of OVAR-3 cells with different concentrations of chitosan-JQ1 nanoparticles.

MTT assay and IC_{50} determination

As displayed in Fig. 3, OVCAR-3 cells were treated with different concentrations (0, 1, 5, 10, 20, and 40 µg/mL) of Ch-J-NPs. To visualize and measure the cell viability after treatment with Ch-J-NPs, MTT was performed. Based on the percent of cell viability for each concentration, IC_{50} was determined at 5.625 µg/mL (Fig. 4).

qRT-PCR

All the samples in 4 groups were evaluated for the expression of genes of interest. To describe the results, the data of qPCR were divided into 4 groups: c-MYC (0.247 ± 0.0094) and hTERT (0.027 ± 0.0082) genes; CDK1 (0.140 ± 0.035), CDK4 (0.031 ± 0.013) and CDK6 (0.001 ± 0.0003) genes; Caspase-3 (0.985 ± 0.8646), Caspase-8 (0.231± 0.1038) and Caspase-9 (0.267 ± 0.065) genes; and BAX (0.327 ± 0.3379) and BCL2 (0.124 ± 0.057) genes (Figs. 5-8). According to Real-time PCR results: the levels of c-Myc were significantly decreased in the tumor + (Ch-J-NPs) and normal Epi + (Ch-J-NPs) groups compared to the tumor group, the expression of c-Myc decreased significantly in tumor + (Ch-J-NPs) group compared to tumor + JQ1 group. The expression of the hTERT was significantly decreased in the tumor + (Ch-J-NPs) group compared to the tumor group. The levels of CDK1 were decreased significantly in tumor + (Ch-J-NPs) and Normal Epi + (Ch-J-NPs) groups compared to the Tumor group. Also, a

decrease in CDK1 expression was observed in the tumor + (Ch-J-NPs) and normal Epi + (Ch-J-NPs) groups compared to the tumor + JQ1 group. The CDK4 gene was downregulated in the tumor + (Ch-J-NPs) and normal Epi + (Ch-J-NPs) groups compared to the tumor group. The CDK6 gene was down-regulated in tumor + JQ1, tumor + (Ch-J-NPs), and normal Epi + (Ch-J-NPs) groups compared to the tumor group. The levels of the caspase 3 gene were significantly increased in the tumor + (Ch-J-NPs) group compared to the tumor group. An increase in the expression of the caspase 8 gene was observed in different groups compared to the Tumor group, but these differences were not statistically significant. The expression of caspase 9 in the tumor + (Ch-J-NPs) group was significantly increased compared to the tumor group. The expression of BCL2 was significantly decreased in tumor + JQ1, tumor + (Ch-J-NPs), and normal Epi + (Ch-J-NPs) groups as compared to the tumor group. BAX expression increased in the tumor + (Ch-J-NPs) group compared to the tumor, tumor+JQ1, and normal Epi + (Ch-J-NPs) groups.

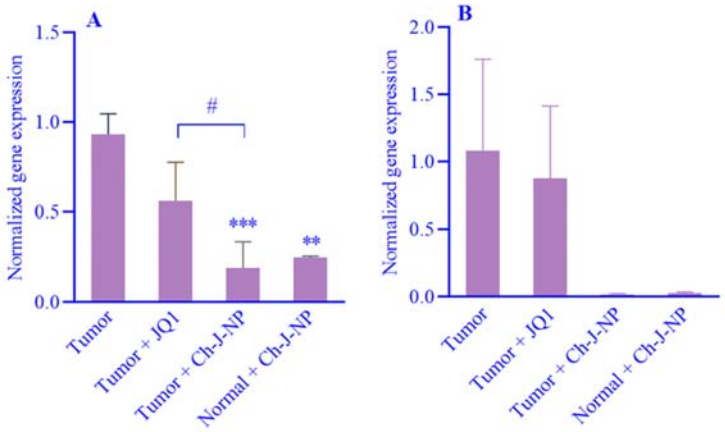

Fig. 5. Real-time polymerase chain reaction analysis of (A) c-Myc and (B) hTERT genes. Data are presented as means ± SD; n = 3. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate significant differences in comparison with the tumor group; $^{\#}P < 0.05$ expresses the difference between the defined groups.

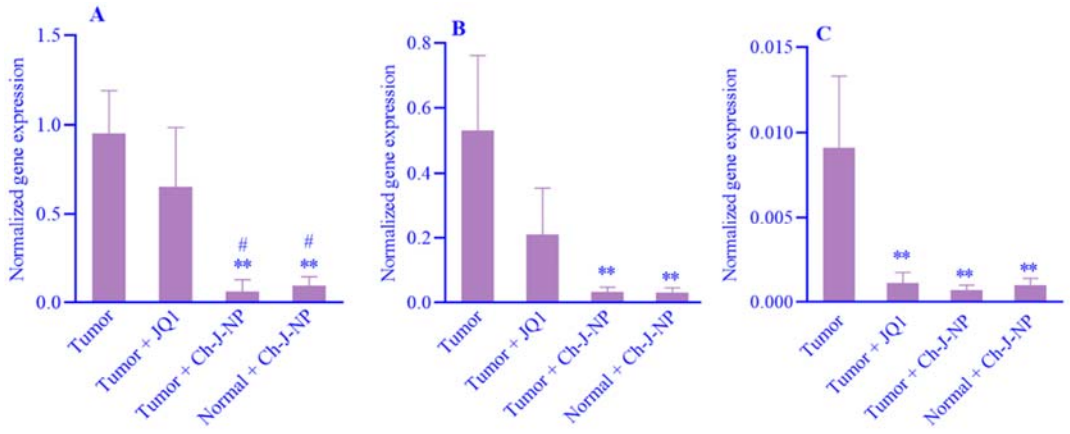

Fig. 6. Real-time polymerase chain reaction analysis of (A) CDK1, (B) CDK4, and (C) CDK6 genes. Data are presented as means ± SD; n = 3. $^{**}P < 0.01$ indicates significant differences in comparison with the tumor group; $^{\#}P < 0.05$ versus tumor + JQ1 group.

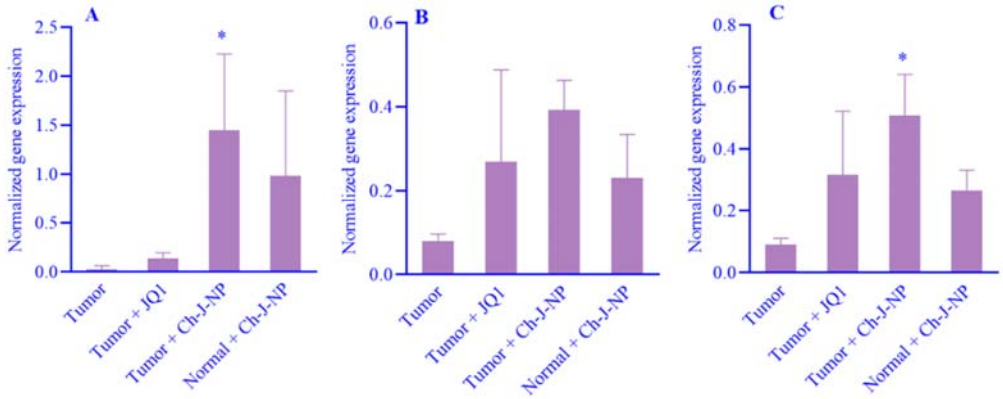

Fig. 7. Real-time polymerase chain reaction analysis of (A) caspase 3, (B) caspase 8, and (C) caspase 9. Data are presented as means ± SD; n = 3. **P* < 0.05 indicates significant differences in comparison with the tumor group.

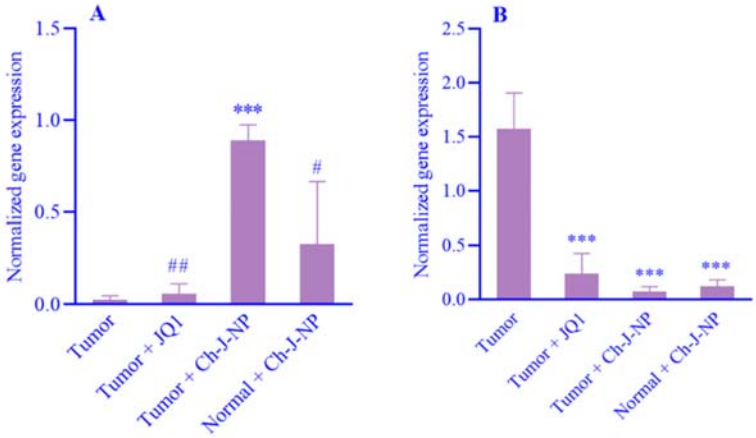

Fig. 8. Real-time polymerase chain reaction analysis of (A) BAX and (B) BCL2 gene expression. Data are presented as means ± SD; n = 3. ***P* < 0.01 indicates significant differences in comparison with the tumor group; #*P* < 0.05 versus tumor + Ch-J-NP group.

DISCUSSION

Chitosan-based nanoparticles have exhibited interesting antitumor efficacy both in vitro and in vivo (30). Chitosan is reported as a potential inhibitor of ovarian cancer (34). BET proteins play major roles in cell cycle regulation at the epigenetic level. JQ1 is a BET inhibitor reagent. It particularly inhibits the function of BRD4 proteins (13). The early research on the effects of JQ1 on hematopoietic malignancies proved that JQ1 targeted BRD4 in hematopoietic cells (52). Later, the influences of JQ1 on solid tumors were also shown (53). Nanoparticles loaded with JQ improved the efficacy against triple-negative breast cancer (54). To study the effects of these two promising approaches (chitosan and JQ1) on OVCAR-3 ovarian cancer cells, we aimed to firstly, load chitosan nanoparticles with JQ1 (Ch-J-NPs); secondly, to evaluate the effects of these nanoparticles on OVCAR3 ovarian cancer cells cycle and apoptosis. To achieve this aim, the expression of 10 important genes related to cell cycle (c-MYC, hTERT, CK1, CKD4, and CKD6) and apoptosis (caspase-3, caspase-8, caspase-9, BAX, and BCL2) was evaluated. In agreement with the above-mentioned reports, the finding of the present study was in favor of cell cycle depression and apoptosis enhancement in OVCAR-3 cells. c-MYC is an oncogene and a master regulator of cell proliferation (37). In malignancies, the function of c-MYC almost is always enhanced through the induction of upstream pathways of c-MYC expression. In ovarian cancer, c-MYC is a key transcription factor (24). It is believed that BET inhibitors act generally as c-MYC inhibitors (55) and there

are several reports about the regulatory effects of JQ1 on c-MYC (56). For instance, JQ1 suppresses the expression of c-MYC in c-MYC-amplified medulloblastoma cells (53). In ovarian cancer, it is suggested that JQ1 suppresses ovarian cancer cell proliferation and promotes apoptosis of these cells by targeting BRD4 and c-Myc (24). In agreement with the mentioned reports, the findings of the present study indicated that c-MYC expression in OVCAR-3 cells significantly decreased following treatment with Ch-J-NPs, compared to the other groups. hTERT is termed a cancer hallmark and is activated in 90% of cancer cells (57). It is reported that hTERT mRNA exists in exosomes derived from tumor cells and can transform non-malignant cells into telomerase-positive ones (38). The role of hTERT in malignancy is so important that its mRNA levels may be an indicator of cancer clinical status and progression of cancer (52). It is believed that c-MYC acts as a direct activator of hTERT transcription (59). In ovarian cancer, hTERT mediates the aggressiveness of ovarian cancer through inducing stress hormones (60). In our experiment, expression of hTERT significantly decreased after treatment with Ch-J-NPs compared to the control group. This finding is in accordance with the results of previous reports. Cyclin-dependent kinases (CDKs) play important roles in the control of cell division and transcriptional regulation (39). CDK1 is the key regulator of the cell cycle and its up-regulation is associated with many malignancies (61). Based on previous studies, CDK1 accumulates in epithelial ovarian cancer cells and can be a prognostic factor in ovarian cancer (62). CDK4/6 is the principal driving factor in cell cycle regulation and plays a key role in the occurrence and progression of various malignancies. CDK4 and CDK6 have 71% amino acid homology, and both can bind to CD1/2/3. Inhibitors of these genes presently are used as therapeutic targets in tumors such as breast cancer (63). In high-grade serous ovarian cancer, CDK4/6 inhibition along with chemotherapy used as maintenance therapy (64). The findings of our study showed that the expression of CDK1, CDK4, and CDK6 decreased significantly following the treatment of OVCAR-3 cells with Ch-J-NPs, which is in agreement with previously mentioned investigations. We also evaluated the expression of three apoptotic caspases including caspase-3 (an executioner caspase) caspase-9 and caspase-8 (initiator caspases). In different types of malignancies, aberrant expression of caspases has been reported (40). It is reported that downregulation of DNA (cytosine-5)-methyl transferase 3α, a suggested anticancer molecule in ovarian cancer, induced caspase-3 and -9-mediated apoptosis (11). It has been shown that depending on the mechanisms that regulate the enzymatic activity of caspase-8, it may show apoptotic and non-apoptotic functions. It is also believed that in gynecological tumors, downregulation or absence of caspase-8 is associated with tumor invasiveness and aggressiveness (41). In our experiment, the expression of all three caspases, caspase-3 and caspase-9, increased significantly after treatment with Ch-J-NPs compared to other groups, which is consistent with previous studies. The Bcl-2 family proteins are the key regulators of apoptosis cell death. BCL2 and BAX, are two members of this family that show opposite functions in the intrinsic apoptotic pathway. BCL2 is anti-apoptotic and BAX is a pre-apoptotic agent. Hence, the high ratio of Bax/Bcl2 is in favor of apoptosis (42,43). A significant enhancement in BAX gene expression, a significant decrease in the expression of the BCL2 gene, and a high ratio of BAX/BCL2 were observed following the treatment of OVCAR-3 cells with Ch-J-NPs.

CONCLUSION

Overall, the results of this study showed that Ch-JQ1-NPs display anti-cell-cycle and apoptosis effects on OVCAR-3 ovarian cancer cells. These effects were remarkably significant compared to non-encapsulated JQ1. The apoptotic effects of these nanoparticles on normal epithelial cells indicate the possibility of undesirable influence on normal cells that must be prohibited. Future studies are needed to investigate and confirm the similar in vivo effects of Ch-JQ1-NPs on ovarian cancer cells.

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Conflicts of interest statement

The authors declared no conflict of interest in this study.

Authors' contributions

M. Soleimani, E. Masoudi, M. Homayoun, M. Bakhtiari, and G. Zarinfard contributed to the study conception and design; M Soleimani, E Masoudi collected and analyzed the data and interpreted the results; M Soleimani wrote the draft of the manuscript. The finalized manuscript was approved by all authors.

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