The p53 Modulated Cytotoxicity of *Ophiocoma scolopendrina* Polysaccharide Against Resistance Ovarian Cancer Cells

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Abstract

**Background:** Marine environment is a valuable source of bioactive compounds with variable medicinal properties. Previously, it was shown that *Ophiocoma erinaceus* extracted polysaccharide has prominent cytotoxic effect on HeLa human cervical cancer cells. In the present study, the anti-cancer properties of polysaccharide extracted from *Ophiocoma scolopendrina* (*O. scolopendrina*) were examined in comparison with paclitaxel as a conventional drug against resistant ovarian cancer; also, its related mechanism against A2780cp ovarian cancer cells was investigated.

**Methods:** The A2780cp cancer cells and NIH3T3 normal cells were cultured and treated with different concentrations of polysaccharide extracted from *O. scolopendrina* for 24 hr and 48 hr. Then, cell toxicity was studied by MTT assay, morphology of cells was observed under inverted microscopy and the type of induced cancer cell death was assessed by annexin-V-FITC, propidium iodide and acridine orange staining. Finally, the apoptosis pathway was determined by measurement of caspase-3 and caspase-9 activity and assessment of p53 and Bcl-2. The statistical analysis was performed by SPSS software, one way ANOVA and p<0.05 was considered significant.

**Results:** Our observations from MTT assay and morphological assessment exhibited that *O. scolopendrina* isolated polysaccharide inhibited proliferation of ovarian cancer cells with IC₅₀ of 35 µg/ml, while paclitaxel suppressed tumor cell growth with IC₅₀=10 µg/ml. In contrast, MTT observations revealed low cytotoxicity of these chemotherapeutic agents against NIH3T3 normal cells. Also, the analysis correlated with induced cell death elucidated that concurrent treatment of polysaccharide plus paclitaxel had a further anti-cancer effect against A2780cp cells mainly through restoration of p53 and mitochondrial apoptosis cell death induction.

**Conclusion:** Taken together, our research supports the finding that application of polysaccharide extracted from *O. scolopendrina* can be considered a promising marine chemotherapeutic approach for advancing efficacy of paclitaxel in treatment of resistant ovarian cancer. Additional *in vivo* experiments are required to elucidate the role of brittle star polysaccharides in animal and clinical trials.

Keywords: Apoptosis, Ovarian neoplasms, Paclitaxel

Introduction

Cancer is a chronic and fatal disease worldwide and ovarian cancer is the fourth women deadly disorder, especially in older women 1,2. Current therapeutic ovarian cancer methods include surgery and the use of chemotherapy is limited due to harmful side effects 3,4. Chemotherapeutic drugs have created multidrug resistance that induces noticeable challenge for oncologists in treatment of cancer 5. To date, several chemotherapeutic drugs have been introduced to interfere with ovarian carcinoma, but among suggested anti-cancer drugs, paclitaxel is a favorable anti-tumor agent which creates cytotoxicity via apoptosis triggering in many tumors, particularly in ovarian cancer 6. Nevertheless, there are unwanted complexities for ovarian cancer treatment 5. Thus, many researches attempted to find novel therapeutic methods to overcome ovarian drug resistance 4,5.

Natural products occupy the main source of chemo-
therapeutic agents in the past few decades that have attracted researchers’ attentions. The low toxicity and minimum levels of side effects in natural products give them priority in comparison to synthetic chemotherapeutic leads. Among various biological effects of natural metabolites, their capacity for inhibition of cancer can be noted as the therapeutic importance.

Polysaccharides are biocompounds that have antioxidant, anti-microbial and anti-coagulant activities. In addition, in recent years, a growing body of investigations concentrated on anti-cancer potential of natural polysaccharides. The used mechanisms by these compounds are tumor growth suppression, apoptosis induction and metastasis prevention. Apoptosis or programmed cell death is a process that is commonly found in living organisms for eradication of redundant or damaged cells. The morphological features in apoptotic cells are shrinkage of cell membrane, DNA fragmentation and apoptotic body generation.

The ocean is composed of tremendous structurally unique marine compounds with biological properties such as anti-microbial, anti-viral, antioxidant and anti-cancer effects. Aquatic echinoderms are accounted as abundant marine invertebrates which contain physiologically active metabolites required in biomedicine.

Among biomedical investigation in echinoderms (composed of Asteroidea: sea stars or starfish, Crinoid: crinoids, Ophiuroidea: brittle stars, Echinoid: sea urchins and Holothuroidea: sea cucumbers), brittle stars (Ophiuroidea) possess unknown bioactive substances rather than starfish (Asteroidea) and sea cucumbers (Holothuroidea) that are related to their therapeutic properties and fewer studies have been conducted on such properties in the last three decades. Brittle star (Ophiuroidea) is an aquatic invertebrate with the capacity for arm regeneration. To date, the presence of some bioactive substances such as terpenes, sulfated sterols, carotenoid sulfate, phenylpropanoids and naphthoquinones in brittle star have been proved which may be important in anti-cancer therapy.

Considering the bioactivity of marine compounds, this study was designed to investigate the concomitant cytotoxic effect of polysaccharide isolated from Persian Gulf brittle star Ophiocoma scolopendrina (O. scolopendrina) and paclitaxel on A2780cp cells and their related mechanism against human ovarian cancer cells.

**Materials and Methods**

**Reagents**

A2780cp cell line (Human epithelial ovarian carcinoma) was purchased from NCBI (National Cell Bank of Iran). Trypsin/EDTA (1X) and fetal bovine serum were provided from Gibco (USA), RPMI-1640 and Trypan blue were purchased from Bio idea (Iran), and penicillin/streptomycin and phosphate buffer saline were purchased from PAA (Austria). PI (Propidium Iodide), DAPI (4’, 6-diamidino-2-phenylindole dihydrochloride) kit and MTT [3-(4, 5-dimethylthiazol-2-yl) 2, 5 di phenyl tetrazolium bromide] and acridine orange/propidium iodide were purchased from Sigma (USA). Annexin V-FITC kit and Caspase-9 assay and Caspase-3 assay kit were prepared from Abcam (UK). Taxol or paclitaxel was purchased from Sigma (USA).

**Extraction of polysaccharide**

Firstly, morphometric estimation of O. scolopendrina was conducted at the Research Center of Applied Biology at Mashhad Branch of Islamic Azad University. Then, specimens were washed and dried in the dark. Then, 500 gr dried brittle star were added to 100 ml water, boiled for 3 hr and centrifuged and filtered. In the next step, 3 volumes of 95% (v/v) ethanol were added and incubated at 4°C overnight. Then, specimen was centrifuged and the precipitate was dissolved in distilled water and centrifuged for 20 min to get aqueous supernatant lyophilized (total polysaccharide).

**Cell culture**

The A2780cp cells and NIH3T3 fibroblast normal cells were cultured in RPMI 1640, DMEM medium with 10% FBS and 1% penicillin/streptomycin in incubator at 37°C containing 5% CO2, respectively.

**MTT assay**

A2780cp cells and NIH3T3 were cultured and treated with different concentrations of extracted polysaccharide from O. scolopendrina (12.5, 25, 50 µg/ml), taxol (5, 10, 20, 40 µg/ml) and synergism treatment was done by brittle star polysaccharide and taxol (12.5 µg/ml polysaccharide +10 µg/ml taxol, 12.5 µg/ml polysaccharide +25 µg/ml taxol) for 24 and 48 hr. After a certain time, the cells were incubated with MTT for 4 hr and DMSO was added to dissolve formazan according to the company’s protocol. Finally, the absorbance of each well was measured at a wavelength of 560 nm by a spectrophotometer.

**Evaluation of induced cell death**

**DAPI staining:** 4’, 6-diamidino-2-phenylindole dihydrochloride staining was used to evaluate the morphology of nuclei. In this assay, A2780cp ovarian cancer cells were seeded on a coverslip and incubated with the desired concentration of brittle star polysaccharide and paclitaxel (IC50) for 24 hr. Then, the cells were washed with PBS and DAPI was added and cells were incubated for 10 min in the dark and morphological changes were observed under the fluorescence microscope.

**Acridine orange/propidium iodide staining**

The apoptotic morphological characteristics of cells were ascertained by acridine orange/propidium iodide staining. The ovarian tumor cells were cultured and treated with IC50 concentration of isolated polysaccharide and taxol and synergism treatment for 24 hr. Then, the cells were washed with PBS and acridine orange (20 µg/ml) and propidium iodide (20 µg/ml) were add-
ed (1:1). Eventually, the cells were observed under a fluorescence microscope.

**Annexin V-FITC-PI**

Annexin/PI method was used to assess phospha
dyserine exclusion on the extracellular side of apopto
tic cells. For this purpose, the A2780cp cells were seed
ed and treated with IC_{50} concentrations of polysaccha
ride and paclitaxel, according to the company’s proto
col. Subsequently, the tumor cells were suspended in 500 μl 1X binding buffer. Thereafter, 5 μl annexin V-
FITC and 5 μl propidium iodide were added and incu
bated for 5 min at room temperature in the dark. Then, they were analyzed using flow cytometer.

**PI staining**

The evaluation of apoptosis was performed using PI
assay. The A2780cp cells were cultured and treated with IC_{50} concentrations of polysaccharide alone or in
synergism state for 24 hr and cell suspension was cen
trifuged and mixed with 700 μl PI solution for 20 min
in the dark. Next, the fluorescence of stained cells was
evaluated using a FACScan laser flow cytometer.

**Caspase-3 and Caspase-9 assay**

Ovarian cells were cultured and treated with anti
proliferative concentrations of isolated polysaccharide
or paclitaxel for 24 hr. Then, cell lysis buffer, 2X reac
tion buffer and LEHD-pNA substrate were added to un
treated and treated cells according to the kit proto
col. At last, the absorbance was read at 405 nm using plate reader.

**Apoptosis related mRNA expression**

The expression of p53 and Bcl-2 mRNA was ana
lyzed by RT-PCR. The total cellular RNAs of ovarian
cancer cells treated with or without extracted poly
saccharide, paclitaxel and combination treatment wereiso
lated using the high pure RNA isolation kit (Roche,
Germany) and reverse transcribed to cDNA using the
easy cDNA synthesis kit (Pars Tous, Iran) according to
manufactures protocol. Then, RT-PCR was performed with 10x buffer, MgCl_2 25 mM, d NTP, and Taq DNA
polymerase. The forward and reverse primer sequence was: 5' CCAGGGCAGCTACGGTTTC 3' forward for
p53 and 5' ACCCTGTCATGCGCTGTGACTG 3' re
verse for p53. The forward and reverse primer se
quences for Bcl-2 were designed as 5' CATGTGTGCT
GAGAGGCAAC 3' and 5' CAGATAGGGCAACCA
GGTGAT 3'. Following amplification, PCR products were
subjected to electrophoresis in a 2% agarose gel.

**Data analysis**

The statistical analysis was performed by SPSS soft
ware, one way ANOVA and p<0.05 was considered
significant.

**Results**

**MTT assay**

MTT analysis showed that treatment with brittle star
polysaccharide at concentration of 30 μg/ml (p<0.01)
could be considered as IC_{50}. Incubation with various
concentrations of taxol exhibited that paclitaxel inhib
ited 50% of ovarian cancer cell growth at 10 μg/ml
(p<0.01). However, treatment with extracted polysaccha
ride and paclitaxel simultaneously exerted more cytoxicity on ovarian cancer cells, so that IC_{50} con
centration was determined as 12.5 μg/ml and 10 μg/ml
(p<0.01) for polysaccharide and taxol, respectively
(Figure 1). Further, there was no significant cytotoxici
ty of brittle star polysaccharide and taxol on NIH3T3
normal cells.

**Morphological observations by inverted microscope**

As exhibited in figure 2, the anti-proliferative activi
ty of brittle star polysaccharide and taxol in IC_{50} con
centration on A2780cp cells induced apparent morp
ho logical alterations such as reduction of cell volume,
cell shrinkage and apoptotic body formation which
induced apoptosis in exposure with cytotoxic dosage of
brittle star polysaccharide and paclitaxel. Meanwhile,
NIH3T3 treated cells didn’t show considerable altera
tions. High quantity of formazan crystals validated the
effect of brittle star polysaccharide and taxol on ovar
ian cancer cells.

**DAPI staining**

DAPI staining was used to study the nucleus mor
phological changes under treatment with isolated poly
saccharide and paclitaxel. As shown in figure 3, the
A2780cp treated cells (By IC_{50} concentration of brittle
stars polysaccharide, paclitaxel and synergist treat
revealed DNA fragmentation which confirmed pro-apoptotic effect of brittle stars polysaccharide and paclitaxel, alone or in combination (Figure 3).

Acridine orange/propodium iodide staining
After treatment, acridine orange/propodium iodide staining was conducted to distinguish apoptosis or necrosis induction under exposure with brittle star polysaccharide and paclitaxel. In this assay, green color is a crucial mark of live cells and red color is indicator of dead or necrotic cells. As shown in figure 4, control group showed green cells; meanwhile in treated cells with IC$_{50}$ concentrations of brittle stars polysaccharide, taxol and simultaneous treatment, more cells were apoptotic, which confirmed involvement of apoptosis in cytotoxicity of brittle stars polysaccharide and taxol and their effect on A2780cp cancer cells (Figure 4).

Annexin V-FITC/PI
According to results obtained by flow cytometric analysis of annexin V-FITC/PI kit, the frequent portion of cell death induced by IC$_{50}$ concentrations of brittle stars polysaccharide and paclitaxel alone or in combination was apoptosis which is indicated in figure 5A.

PI assay
In PI assay, apoptotic cells showed a sub-G1 peak. Results from this assay showed that brittle stars polysaccharide and taxol in IC$_{50}$ dosage evoked apoptosis in A2780cp ovarian tumor cells (Figure 5B).

Caspase-3 and caspase-9 colorimetric assay
Caspase-3 and caspase-9 colorimetric assays were conducted to determine the direction of apoptosis induced by polysaccharide and paclitaxel. The results showed that the induced apoptosis pathway by desired dosage of brittle stars polysaccharide, taxol and concurrent treatment of them was caspase-dependent indicating the intrinsic pathway induced by brittle star polysaccharide and paclitaxel (Figure 6).
Cytotoxicity of *Ophiocoma scolopendrina* Polysaccharide Against Resistant Ovarian Cancer Cells

**p53 and Bcl-2 expression**

The analysis of transcriptional levels of two apoptotic-related genes, p53 and Bcl-2 in human ovarian cancer cells incubated with IC\textsubscript{50} concentrations of brittle star polysaccharide and paclitaxel showed that pretreatment of A2780cp cells with polysaccharide, taxol and synergistic treatment downregulated Bcl-2 expression and upregulated p53 mRNA level (Figure 7).

**Discussion**

In our study, the cytotoxic effect and the type of cell...
death induced by polysaccharide extracted from brittle star *O. scolopendrina* were investigated and its concurrent effect with paclitaxel against ovarian cancer cells was evaluated. A2780cp cell line is a human epithelial ovarian carcinoma and taxol is used as a usual, approved and appreciable chemotherapeutic drug for treatment of some cancers like ovarian cancer. This agent was used in this study (As positive control) to assess anti-cancer efficacy of the brittle star polysaccharide against ovarian cancer cells.

So, the single and synergistic effect of brittle star polysaccharide and paclitaxel on A2780cp human ovarian cancer cells was examined. The morphological observation and MTT assay exhibited that the derived polysaccharide induced an anti-growth effect (IC$_{50}$=30 µg/ml) in a dose-time dependent manner on A2780cp cells and taxol exerted 50% cytotoxicity in concentration of 10 µg/ml. The PI, annexin V-FITC, DAPI and acridine orange/propidium iodide assay showed that the combination treatment of *O. scolopendrina* polysaccharide and taxol induced more apoptosis. Besides, the conducted experiments using caspase-3 and caspase-9 enzymatic activity showed that the apoptosis induced by brittle star extracted polysaccharide and paclitaxel in A2780cp cells was caspase-dependent or related to mitochondrial pathway. As activation of p53 stimulates apoptosis, up-regulation of p53 and down regulation of Bcl-2 under treatment with *O. scolopendrina* polysaccharide and taxol may be an effective mechanism of cell death in resistance of A2780 cells.

Results obtained in other studies related to the biological characteristics of the natural polysaccharides, confirmed the anti-cancer effect of these compounds. Chen *et al* in 2013 documented medicinal properties of polysaccharide extracted from fungus *Rhizopus nigricans* and revealed that this isolated polysaccharide inhibited human gastric cancer cell growth -BGC-823 using apoptosis increment via mitochondrial mediated pathway 20.

Lavi *et al* in 2006 reported anti-tumor effect of polysaccharide fraction from mushroom *Pleurotus ostreatus* on HT-29 cell line 21. Cao *et al* exhibited that *Angelica sinensis* polysaccharide induced apoptosis via intrinsic pathway in HeLa cervical cancer cells *in vitro* and *in vivo* 22.

In addition, there are evidences on the basis of utilization of natural polysaccharides from flora of terrestrial ecosystem in biomedicine 10,11,23. Gamal-eldien in 2009 evaluated the biological effect of various fractions of polysaccharide from brown algae *Sargassum latifolium* and proved that E3 fraction indicated anticancer activity against leukemia cells as compared with E1, E2 and E4 fraction 24. Lee *et al* in 2011 examined the anti-metastatic potential of polysaccharide extracted from *Asterina pectinifera* in MDA-MB-231 breast cancer cells and displayed that extracted polysaccharide had tumor growth inhibitory effect on examined breast cancer cells 25.

Lu *et al* in 2012 demonstrated the tumor inhibitory effect of *Coix lacryma-jobi* (Adlay seed) polysaccharide fraction against A549 cancer cells and reported that intrinsic apoptosis pathway was responsible for this cytotoxicity 26. In 2014, Wang *et al* reported that *Boschniakia rossica* polysaccharide suppressed Hep2 cell line proliferation with G0/G1 cell cycle arrest and elicited reprogrammed cell death through mitochondrial pathway 27. Furthermore, Thangam *et al* displayed the growth inhibitory effect of polysaccharide fractions extracted from *Cymbopogon citratus* against LN-cap and SiHa tumor cells 28.

p53 is a tumor suppressor and a nuclear transcription factor which exhibits loss of function mutations in part of cancer cell types. Existence of mutation in p53 conferred chemo-resistance phenotype in malignant cells. Oncological studies showed that accumulation of p53 in tumor cell nucleus elicits pro-apoptotic activity 29. Yazdanpanahi *et al* elucidated that increase in p53 gene expression is a molecular mechanism responsible for natural compound cytotoxicity in breast cancer cells 30. In a previous study, it was indicated that *Ophiocoma erinacea* (*O. erinacea*) polysaccharide can be an appropriate cytotoxic compound against human cervical cancer cells 31. Consequently, all experiments performed in this field in agreement with our observations confirmed the importance of natural polysaccharides in the treatment of lethal diseases such as cancer.

**Conclusion**

In this study, the anti-cancer effect of extracted polysaccharide from brittle star *O. scolopendrina* and taxol (chemotherapeutic drug) alone and in combination was evaluated against resistant ovarian cancer *in vitro*. According to our observations, polysaccharide extracted from *O. scolopendrina* and paclitaxel induced apoptosis cell death *via* intrinsic caspase-dependent pathway in ovarian cancer cells. It can be suggested that this marine polysaccharide was a worthy anti-cancer candidate to increase the anti-tumor efficacy of paclitaxel for treating human ovarian cancer, but additional investigations are needed to assess clinical oncological studies.

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**Conflict of Interest**

All of the authors declared no conflict of interests.

**References**

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17. Czarkwiani A, Dylus DV, Oliveri P. Expression of ske-