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Research Article

Do derived whitecheek shark proteins motivate T cells to fight cancer cells? A case study in using SW742 cell line

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Abstract

Shark cartilage is considered as a natural dietary supplement consisting of anti-angiogenic, immunostimulatory, and anti-inflammatory characteristics. Therefore, this study was designed to inspect the possibility that whitecheek shark (*Carcharhinus dussumieri*) cartilage proteins motivate expression of NKG2D, CXCR3, NKP46, and NKP44 receptors on natural killer cells and their activities against SW742 cell line. To this end, cartilaginous areas of whitecheek shark were caught, minced, stored at −20°C and then lyophilized and kept in refrigerator. Peripheral blood samples were collected from healthy people and a number of 10^6 cells were considered for cell viability using trypan blue method. Activation of NK cells was determined using 10^6 cells stimulated following exposure to 0.2 and 3 mg mL^{-1} extracted shark cartilage proteins in 4, 8, and 18 h incubation. The results showed that cytotoxicity of NK cells increased significantly by elevating the concentration of extracted proteins at incubation period of 24 h (p<0.05). The findings demonstrated that NK activity elevated markedly by increasing the concentration and volume of protein suspension and the exposure time (p<0.05). Interestingly, the expression of NKG2D, CXCR3, NKP44, and NKP46 receptors was not significant in any transcription level by exposing to 0, 25, and 75 µg mL^{-1} in 4, 8, and 18 h incubation period (p>0.05). Together, extracted shark cartilage protein could motivate the immune system capabilities through using NK cells against cancer tissues but specific receptors on T cells cannot be activated by these bioactive materials.

Keywords: Whitecheek shark, NK cells, Cytotoxicity, NK activity, Gene expression, Derived proteins.

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Introduction

During the last few decades, marine chemistry has discovered and exploited thousands of new chemical and medical compounds from aquatic ecosystems, especially the oceans (Fenical and Jensen, 2006; Gerwick and Moore, 2012; Mehbub *et al.*, 2014). The exploitation for healing medical disorders began in the Age of Antiquity and initially documented by the use of marine invertebrates in ancient Greece (Florean *et al.*, 2020). Tremendous variety of chemical structures found in marine biota is attributed to the wide range of environmental conditions and in turn their high genetic diversity (Hay, 2009).

Although marine chemistry discovered thousands of natural compounds, so far only a fraction of marine origin molecules are approved for clinical use (Rangel and Falkenberg, 2015; Jiménez, 2018; Dyshlovoy and Honecker, 2020). Among them, five compounds, including cytarabine, eribulin mesylate, trabectedin, brentuximab vedotin, and ziconotide are marketed for cancer therapy (Nigam *et al.*, 2019; Jimenez *et al.*, 2020). Dozens of shark-cartilage-derived products are introduced to the public as food supplements for the treatment of degenerative diseases such as cancer, arthritis, osteoarthritis, systemic sclerosis, and neurovascular glaucoma in humans and animals (Hammerness *et al.*, 2002).

Some equivocal scientific evidence is published concerning the concept that shark cartilage may have the potential to inhibit the growth of solid tumors (González *et al.*, 2001; Horsman and Van der Kogel, 2009). Literature have already reported the overexpression of angiogenesis inhibitors in shark cartilage and subsequent inhibition of solid tumors (Horsman *et al.*, 1998; Sakkas and Platsoucas, 2007). This antiangiogenic property led researchers to isolate these inhibitors and suggest their use in applied studies aiming to create treatments for cancer and other malignant diseases in humans (Hammerness *et al.*, 2002). On the other hand, other scientists have attributed the media and public attention to shark cartilage as a cancer cure to a visceral fear of cancer and stated the failure of phases I/II clinical trial in supporting the beneficial effect of shark cartilage (Miller *et al.*, 1998; Loprinzi *et al.*, 2005). Medical researchers have attempted seriously to find green ways to remedy cancer diseases because of detrimental side effects of chemotropic treatments to patients (Subramaniam *et al.*, 2019).

Whitecheek shark (*Carcharhinus dussumieri*; Müller and Henle, 1839) is a requiem shark of the family Carcharhinidae. It is native to Indo-Pacific region, from the Arabian Sea and Persian Gulf to Java Indonesia, Japan, and Australia (Safari *et al.*, 2015; Tokunaga *et al.*, 2018). The shark usually is caught as bycatch rather than as the target species and in Australian waters makes up around 2 to 3% of the total biomass caught. Skin and cartilage of whitecheek shark are a major portion of the fishery waste, particularly in

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production of shark fins and fillets (Safari *et al.*, 2015).

Extracted shark cartilage can induce various immunomodulatory and stimulatory effects on immune system response through enhancing the expression of destructive genes and, in turn, increasing the cytotoxic activity of natural killer (NK) cells. Hence, this study seeks to obtain data which could help to mitigate controversial ideas about the shark cartilage extract as a potential cure for cancer and possible underlying mechanism. Moreover, using bioactive compounds derived from aquatic species to remedy colon cancer disease was one of the most important initiatives which was emerged to this research. To this end, the indirect cytotoxicity of the protein fractions derived from whitecheek shark (*Carcharhinus dussumieri*) cartilage to SW742 cells (a colorectal cancer cell line as target cell) was assessed through measuring the expression of NKP44, NKP46, and NKG2D genes on mononuclear cell.

Materials and methods

Shark cartilage sampling

Cartilaginous areas of whitecheek shark caught from Persian Gulf were separated, minced and frozen at −20°C. The samples were lyophilized using freeze dryer and then minced, grinded and sieved through 170 µm mesh. The sieved cartilage kept in a refrigerator until start the experiments.

Cartilage extraction and protein isolation

The cooked cartilage was homogenized in sodium acetate buffer (0.1 M, pH 5.8). Briefly, guanidine hydrochloride (4 M) and two antiproteases, including ethylene diamine tetra acetic acid (EDTA) and phenylmethylsulfonyl fluoride (PMSF) were added to the buffer solution. A total of 1g of cooked cartilage was homogenized in 10 mL of the buffer at 40°C for 48 h. The suspension was centrifuged at 4000 rpm for 30 min and the supernatant was taken and stored at -20°C. About 100 mL of the collected supernatant was mixed with 20g of anhydrous polyethylene glycol (PEG6000), incubated at 4°C for 24h, and centrifuged at 4000 rpm for 45 min. Then, the precipitated substance was dissolved in phosphate buffered saline (PBS, pH: 7.4) and dialyzed (Mw cut off 3.5 kDa) at 4°C for overnight. The protein fractions within the dialyzed solution were precipitated and isolated using ammonium sulfate.

Cartilage protein concentration

To quantify protein abundance in the isolated cartilage protein complex, Bradford protein assay was applied (He, 2011). Corresponding vice versa volumes of PBS and albumin bovine serum (10, 30, 50, 70, and 90 µl) were added into five test tubes, and then about 5 mL of an as-papered reagent (100 mg coomassie brilliant blue G-250+95% ethanol+88% phosphoric acid) was added and vortexed. The absorption spectra of the solutions were scanned at 595 nm using a Spectra-MAX-PLUS

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384 UV–visible spectrophotometer (Molecular Devices, USA) and the calibration curve (r^2=0.995) was drawn to measure the concentration of extracted protein.

Preparation of peripheral blood mononuclear cell (PMBC)

Isolation of peripheral blood mononuclear cells (PBMCs) was carried out according to Sheikhi *et al.* (2008). For this, fresh blood was collected from healthy people and mixed with equal volume of PBS (Núñez *et al.*, 2017). Moreover, the incubation of peripheral blood mononuclear cells was conducted until starting the experiments. The diluted blood was poured into a falcon tube containing Ficoll-Paque media and centrifuged at 2500 rpm for 20 min at 4°C. Then, the separated layer of PBMCs was harvested and washed three times with PBS. The desired cells were suspended in 10% RPMI-FBS medium and their viability was determined using the following formula:

$$\text{Cell viability (\%)} = \frac{\text{Viable cells}}{\text{Dead cells}} \times 100$$

Finally, MTT assay was performed with 1×10^6 cell mL^{-1}.

Activation of natural killer cells

For activation of NK cells, the procedure reported by Sheikhi *et al.* (2014) was conducted in all steps of the experiment. A small amount of the collected blood was mixed with equal volume of PBS (0.2 M and pH 7.4). The NK cells were separated using Ficoll-Paque media at 900g centrifugation for 15 min and the suspension was incubated at 37°C for 2 h. These cells (106) were used as effectors for the experiment and stimulated following exposure to different doses (0.2 and 3 mg mL^{-1}) of protein fraction extracted from the shark cartilage. Activation process was conducted in 96-well microplates at 37°C in 4, 8, and 18 h.

SW742 cell culture

The cell line was prepared from Razi Vaccine and Serum Research Institute, Iran. The cells were cultured in RPMI-1640 medium (FBS, 5% v/v; streptomycin, 100 mg mL^{-1}; penicillin, 100 mg mL^{-1}; and L-glutamate, 2 mM) with 5% CO2 and 95% O2 at 36°C. Trypsin was used to detach the cells, and the suspension was diluted with PBS to deactivate trypsin, and centrifuged at 18000g for 5 min. The precipitated SW742 cells were collected.

Cytotoxic activity of natural killers

Stimulatory influence of the protein fractions on cytotoxicity of NK cells against SW942 cells was evaluated using MTT assay. Briefly, three ratios of activated NK cells to SW742 cells (10:1; 25:1; and 50:1) were poured into the micro plates. PBS was used as negative control. The cells were cultivated (at 37°C; with 5% CO2) for 3 days and exposed to 25 µL MTT per well (for 4, 8, and 18 h during exposure time. The supernatant was collected, mixed with 100 µL DMSO, and read with ELISA at 540 nm. Activity of NK cells was determined using the following formula:

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$$\text{NK activity (\%)} = 1 - (\text{OD (E + T)} - \text{ODE})/\text{ODT} \times 100$$

Where ODE and ODT are optical density values of NK cells and SW742 cells, respectively.

Cytotoxicity of cartilage-derived proteins

Direct cytotoxicity of the cartilage proteins to SW742 cells was assessed using MTT assay. A total of 2×10^4 SW742 cells were exposed to different concentrations of the extracted proteins (0.2 and 3 mg mL^{-1}) in 96-well microplates. The cells were incubated at 37°C for 4 h and then 10 µL of MTT was added into each well and incubated again for 4 h. A solubilisation solution containing 120 µL KOH (2 M) and 140 µL DMSO was added to each well and the cells were incubated for 24 h. The cell suspension was centrifuged at 900g and 0.1 mL of the suspension was transferred into ELIZA microplates. Absorbance of the plates was measures at 550-600 nm and cytotoxicity of the extracted proteins was determined as follows:

$$\text{Cytotoxicity (\%)} = (\text{OD Ncontrol} - \text{Ntest})/(\text{OD Ncontrol}) \times 100$$

Where OD Ncontrol and OD Ntest are optical density values control and treated SW742 cells, respectively.

RNA extraction and relative mRNA expression

Transcription of NKP44, NKP46, and NKG2D genes by mononuclear cell chemokine receptor CXCR3 by lymphocytes was measured according to the established method. Total RNA was extracted using 1 mL Trizol Rigent kit (Jena Bioscience GmbH, Jena Germany) according to the manufacturer instructions previously applied in similar experiments (Groom and Luster, 2011; Hormozi *et al.*, 2017). Quality and quantity of total RNA were assessed by 1% agarose gel and spectrophotometer (at 260 nm and 260/280 nm), respectively. cDNA was synthesized using First Strand cDNA Synthesis kit (Thermo Fisher Scientific) according to the manufacturer's instruction. To do the experiment, a complete cDNA kit was applied to prepare the first strand cDNA from RNA templates supplied with both oligo(dT)18 and random hexamer primers. The oligo(dT)18 primer anneals selectively to the poly(A) tail of mRNA. Random hexamer primers do not require presence of the poly(A) tail, therefore, they can be used for transcription of the 5'-end regions of mRNA or cDNA synthesis of RNA species lacking a poly(A) tail (e.g., microRNAs). qPCR reactions were run in triplicate using a standard protocol as follows: initial denaturation at 95 °C for 30 secs, 40 cycles of denaturation, annealing and extension at 57 °C for 15s. Real time quantitative PCR (RT-qPCR) experiment was performed using Takara SYBR Premix Ex Taq (Takara, Dalian,

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China) to quantify the genes' transcription. Data was analyzed as previous reported by Relative Expression Software Tool for Rotor-Gene (REST-RG, version 3). Total RNA from primary cortical cells was isolated using High Pure RNA isolation kit (Roche). Briefly, cells were seeded at density of 1.5×10^6 cells per well in 6-well plates, followed by pre-treatments with phospholipids and CORT insult. Then, cells were homogenized in lysis buffer and transferred into filter tubes provided in the kit. Following procedure was performed according to manufacturer instructions. RNA concentration was determined using ND-1000 spectrophotometer and reverse transcription was assessed using ExiLERATE LNA™ qPCR, cDNA synthesis kit (Exiqon). Subsequently, PCR reaction was performed using ExiLERATE LNA™ qPCR SYBR green master mix kit (Exiqon) in a lightcycler 480 II (Roche). Each sample was analyzed in triplicate for both target gene and reference gene (β-actin), and relative mRNA expressions were calculated using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen, 2001).

Statistical analysis

The data was analyzed using SPSS software (version 22). All results are presented as mean ± standard deviation (S.D.). Kolmogorov–Smirnov test was applied to assess normality and homogeneity of data. Significant differences were determined using one-way ANOVA, followed by Duncan's test to compare the differences among the experimental groups and the respective control group. Differences were considered statistically significant when $p<0.05$.

Results

Protein purity

To identify the isolated protein, we run the fractions of protein on SDS-PAGE. Applied SDS-PAGE electrophoresis method displayed the extracted protein fractions from the shark cartilage (Fig. 1). According to SDS-PAGE analysis, crude extract represents several low- and high-molecular weight proteins. The purified protein derived from shark cartilage appeared as a single protein band on the SDS gel, with molecular weight of 14.5 kDa.

Gene expression

Expression of NKG2D, CXCR3, NKP46, and NKP44 are presented in Figure 2. Surprisingly, these genes showed no difference in transcription level. Indeed, after 4, 8, and 18 h incubation, the expression of NKG2D exhibited no significant change following exposure to 25 and 75 µg mL^{-1} of the extracted protein. Similarly, CXCR3 demonstrated identical results through using 0, 25, and 75 µg/mL protein suspension in activation of these receptors. Furthermore, both NKP46 and NKP44 receptors had no significant reaction in exposing to 25 and 75 µg mL^{-1} protein suspension in comparison with the control group.

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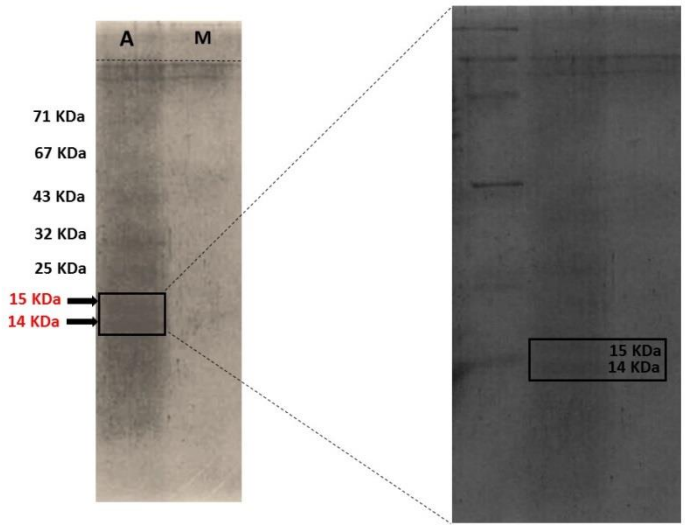

Figure 1: SDS-PAGE electrophoresis of protein fractions extracted from shark cartilage. A, B, C, and D are extracted protein fractions; the main fraction placed on 14.5 KDa.

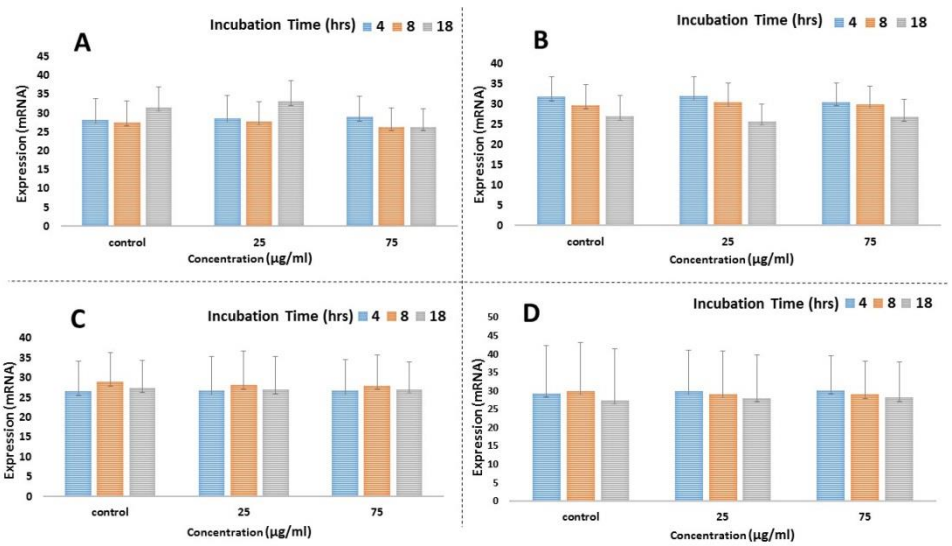

Figure 2: Expression of NKG2D, CXCR3, NKP46, and NKP44 (A, B, C, and D respectively) in 4, 8 and 18 hours after exposing to 0,25 and 75 µg/ml shark cartilage protein.

Cytotoxicity

The results of cytotoxicity test are showed in Figure 3. Overall, the concentration of 0.2 mg mL^{-1} demonstrated the lowest percentage of cytotoxic activity while after 24 hours 3 mg mL^{-1} represented the highest cytotoxicity. Moreover, cytotoxicity index revealed a concentration- and time-dependent pattern as this factor increased significantly at 3 mg mL^{-1} after 24 hours. In regard to incubation time, both 4 and 8 hours unfolded as same cytotoxic activity but by 24-hour incubation cytotoxicity of protein suspension was considerably significant in 0.2 and 3 mg mL-^{1}. In terms of protein volume, results showed that in 3 mg mL^{-1} all volume had the highest cytotoxicity because of the greatest amount of protein compared to 0.2 mg mL^{-1}.

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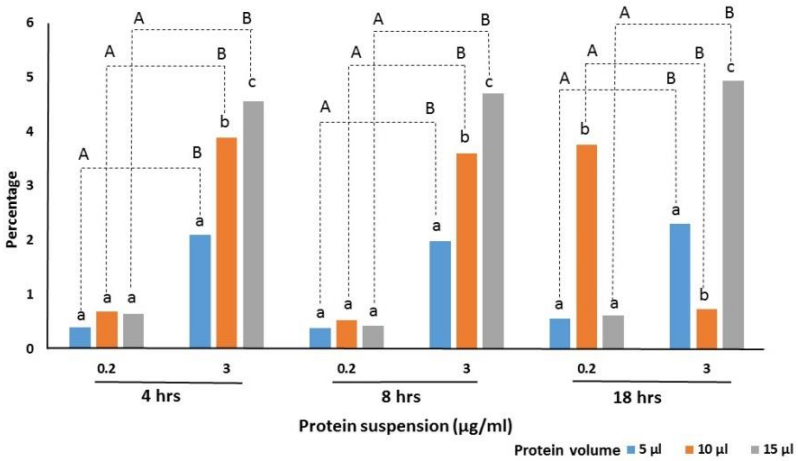

Figure 3: Cytotoxic activity of protein fractions derived from shark cartilage in 5, 10 and 15 µL volume and 0.2 and 3 mg ml-1 concentration after 4, 8 and 18 hours of incubation.

NK activity

Stimulating impacts of protein fractions derived from shark cartilage are presented in Figure 4.

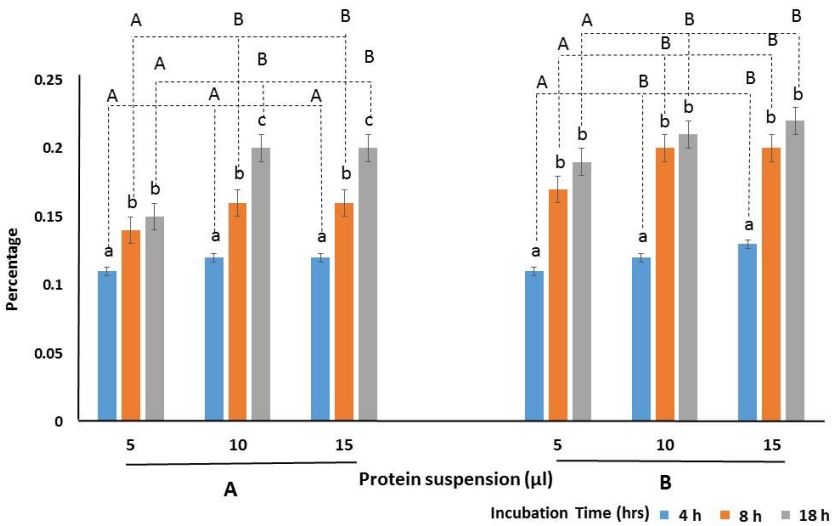

Figure 4: NK activity of protein fractions derived from shark cartilage in 5, 10 and 15 µL protein suspension after 4, 8 and 18 hours of incubation (A: 0.2 and B: 3 mg ml^{-1} protein concentration).

In general, the results showed a concentration-, time- and volume-relevant response as the activity of NK cells increased significantly. Following the first 24-hour incubation time, NK activity revealed no considerable change among concentrations and volume of protein suspension except 3 mg mL^{-1}

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with 10 and 15 µL that showed a slight change compared to 5 µL. After 48 hours of SW742 cells incubation, although both concentrations (i.e. 0.2 and 3 mg mL^{-1}) in all volumes of protein suspension demonstrated no significant alteration, 5 µL could motivate NK cells in comparison with other volume; in this exposure time NK activity was elevated significantly compared to 10 and 15. By exposing SW742 cells to extracted shark cartilage over 72 hours, NK activity in 10 and 15 µL containing 0.2 mg mL^{-1} protein was higher than the volume at 5 µL, whereas 5, 10, and 15 µL consisting of 3 of mg mL^{-1} protein revealed no significant difference in NK activity.

Discussion

Some investigations have been conducted to characterize numerous pharmaceutical substances in shark cartilage which avoid getting cancer (Lagman and Walsh, 2003; Molaie *et al.*, 2019). Several studies supported the hypothesis that shark cartilage is a source of antiangiogenic and antitumor compounds (Gingras *et al.*, 2000; González *et al.*, 2001; Kang *et al.*, 2003). Capability of extracted shark cartilage proteins in strengthening and stimulating the immune cells is proven in countless studies carried out in cancer research (Sheikhi *et al.*, 2014). The purity of protein extracted from shark cartilage is more important in terms of focusing on the main fractions of protein as Molaie *et al.* (2019) mentioned in their study who evaluated the synergistic therapeutic effect of these healing compounds on mice. Our findings in SDS-PAGE analysis showed that the main fraction of protein places on 14.5 kDa protein band in gel which was higher than the results of Rabbani-Chadegani *et al.* (2008); these authors reported that the main fraction of shark cartilage protein place on 13.7 kDa. Comparatively, researchers in several investigations reported 14.5 KDa for the main base pair extracted protein used in cancer studies (Hassan *et al.*, 2005; Rabbani-Chadegani *et al.*, 2008). Similarly, Safari *et al.* (2015) claimed that shark cartilage protein fraction with the most immuno-stimulatory effects is composed of two proteins with molecular weights of about 14 and 15 kDa which is fairly close to our results.

Diversity within NK cell repertoire is achieved through the processes of education and maturation, which determine the specific combination of receptors expressed on the cell surface of each NK cell and ensure self-tolerance (Cheent and Khakoo, 2009; Carrillo-Bustamante *et al.*, 2016; Freud *et al.*, 2017). Inhibitory receptors play a well-established role in NK cell education, but the role of activating receptors in this process has been yet remained in the dark (Anfossi *et al.*, 2006; He and Tian, 2017; Pfeifer *et al.*, 2018). In this regard, NK cell activating receptors including NKG2D, NKP46, NKP44 and CXCR3 were considered as required genes to show the activation status of T-cells. Surprisingly, extracted shark cartilage protein could not significantly motivate these receptors and in turn the expression of them exhibited no significant change in the level of

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transcriptions. In other words, even 75 µL/mL shark cartilage protein coupled with the longest duration of SW742 incubation (i.e. 18 hours) showed no considerable rate of expression among genes. Literature have illustrated that NKG2D is not essential to NK cells development and maturation as well as CXCR3 that could not be stimulated by shark cartilage protein (Muntasell *et al.*, 2010; Shahiri Tabarestani *et al.*, 2016; Le Gars *et al.*, 2019). In another study Sheppard (2013) reported that two activating receptors NKG2D and NKP46 in natural killer cells could not be activated through using different concentration of shark cartilage protein which is consisted with our results. Kuo *et al.* (2018) claimed that CXCR3 responded quickly to the shark cartilage protein because homeostatic proliferation of T cells in immune depleted individuals can lead to an enrichment of CXCR3+T cells. Moreover, the expression of CXCR3 showed an indirect time-related response to shark cartilage protein as previously described by Metzemaekers *et al.* (2017) who investigated the regulation of CXCR3 ligands at distinct levels. CXCR3 is a chemokine receptor that is highly expressed on effector T cells and plays an important role in T cell trafficking and function (Colvin *et al.*, 2004; Kuo *et al.*, 2018). This receptor could be activated by three interferon-inducible ligands, including CXCL9 (MIG), CXCL10(IP-10) and CXCL11 (I-TAC) for immune activation against cancer cells; that is, shark cartilage may contain these ligands affecting CXCR3 receptors and in turn leading to increasing the number of natural killer cells (Sridhar and Shepherd, 2003; Hassan *et al*., 2005; Groom and Luster, 2011). Interestingly, in our investigation, CXCR3 did not respond positively to exposing by extracted proteins and this receptor could not be affected by shark cartilage proteins. A study carried out by Karin (2020) revealed that CXCR3 receptors activated by exposing to shark cartilage proteins because of the immune system response. As natural cytotoxicity receptors, including NKP46 and NKP44 play a crucial role for natural killer (NK) cell functions, they are acquired early during development from NK precursor cells to mature NK cells (Barrow *et al.*, 2019). The results of this study yielded information pertaining to expression of NKP46 receptors in 4, 8, and 18 hours of SW742 incubation by exposing to shark cartilage proteins, yet findings demonstrated no significant difference among concentrations and times. Studies have shown that extracted shark cartilage proteins have no considerable impact on stimulating NKP46 receptors resulting in increasing the number of T cells in mammalian blood circulation system (Bargahi et la., 2011). As the natural cytotoxicity receptor NKP44 bind with a specific ligand inducing powerful NK cell activation and cytotoxicity, this gene is flawless and significant due to its restriction to activated NK cells and killing of many tumor cell lines (Rosental *et al.*, 2011; Rajagopalan and Long, 2012; Horton and Mathew, 2015; Parodi *et al.*, 2019).

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According to the results of our study, expression of NKP44 represented no significant difference among concentrations and times. Physiologically, tumors may also exploit NKP44 to escape NK cell recognition, but NK cells utilize NKP44 to recognize and kill targets (Sheikhi *et al.*, 2014). Based on the findings, it is clear that NKP44 could not be expressed by different concentrations of extracted shark cartilage proteins.

Percentage of cytotoxic activity of NK cells showed that in 3 mg mL^{-1} of extracted shark cartilage protein the highest severity of cytotoxicity was occurred compared to 0.2 mg mL^{-1}. In other words, our findings exhibited that SW742 cell line showed a concentration-related response through cytotoxicity of extracted shark cartilage protein. Based on the results, certain proteins from shark cartilage can augment and stimulate the cytotoxic activity of NK cells against SW742, as target cells in this experiment. These findings are consisted with the outcomes reported by Bargahi *et al.* (2011) who investigated the effects of shark cartilage derived protein on NK cells activity. The most important reason for this phenomenon can be the expression of cytokines, especially IL-2, and IFN-γ, that activates the natural killer (NK) cells cytotoxicity (Sheppard, 2013). Moreover, by incubating SW742 cell line after 4 and 8 hours there was no significant change between concentrations (i.e. 0.2 and 3) but 18-hour exposure period revealed different levels of cytotoxicity. In this regard, it is argued that special bioactive materials in shark cartilage can affect the immune system after some hours, so in our study it is possible that the cytotoxicity response of SW742 cells may be occurred after 18 hours of exposure (Hammerness *et al.*, 2002). In terms of different volume of protein suspension used in cytotoxicity test, the results showed no significant difference after 4 and 8 hours in 0.2 mg mL^{-1}, yet this index increased significantly in 3 mg mL^{-1} in the given times. These findings suggested that extracted shark cartilage proteins showed a dose-dependent pattern instead of a time-related manner as with the elevation of concentration colon cancer cells underwent significant levels of cytotoxicity. After 18-hour incubation, only volumes of 10 and 15 µL (in 0.2 and 3 mg mL^{-1}, respectively) demonstrated different levels of cytotoxicity. These results also demonstrated that volume and concentration of derived shark cartilage proteins had higher levels of cytotoxicity against SW742 cells.

According to the results, the NK activity showed both time- and volume-related response to cartilage proteins (in 0.2 mg mL^{-1}) as the activity of natural killer cells increased significantly after 18 hrs incubation time compared to the first and second 18-hour exposure period. The most important physiological reason relating to this occurrence would be that such proteins have a broad array of chemotherapeutic properties and immunosuppressive strategies which foster immune evasion and prevent host immune cells

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(Horsman and Van der Kogel, 2009; Le Gars *et al.*, 2019). Exposing to 3 mg mL^{-1} extracted protein, NK activity increased through elevating the incubation period and volume of protein suspension; in turn, the concentration, exposure time and volume of proteins had a direct impact on increasing activity of NK cells. In a similar study, Hassan *et al.* (2005) reported that intraperitoneal injection of these protein fractions to tumor-bearing mice could increase T-cell infiltration into the tumor, and decrease the tumor lesion size that finally increased the activity of natural lethal cells. It has been proven that NK cells possess specific receptors on their surface for certain shark cartilage derived proteins; therefore, NK cells can be capacitated directly with these proteins to fight cancer cells (Ben-Shmuel *et al.*, 2020). Many studies have shown that in patients with cancer, NK cells possess no efficiency against tumors and virus infections as the first line of defense system in body (Li and Sun, 2018). In addition, NK cells are a reliable source of innate immune cells participating in managing pathological situations like tumors and viral infections as well as some physiological conditions, such as pregnancy (Hammerness *et al.*, 2002; Le Gars *et al.*, 2019; Olmos-Ortiz *et al.*, 2019). Findings of the present study demonstrated that the lowest NK activity occurred in the first 24 hours in both concentrations (0.2 and 3 mg mL^{-1}), which can be due to a delay for immune system response (Hassan *et al.*, 2005). These results are lined with the findings, which reported that it takes hours for activation of T cells exposed to shark cartilage proteins, especially for motivation of humoral immune system factors (Hammerness *et al.*, 2002). Exposing SW742 cells to extracted shark cartilage proteins target effector cells in 5, 10, and 15 µL and 0.2 and 3 mg mL^{-1} concentrations underwent the highest NK activity after 72 hours compared to 24 and 48 hours. This is because inhibitory factors that have negative impacts on NK cells activities, such as macrophages and prostaglandins, could lead to reducing the activity of NK cells in the first hours of incubation process; consequently, as exposure time increased to 48 and 72 hours, with decrease in these factors the NK activity increased (Kalinski, 2012).

The current study attempted to assess the impacts of derived protein compounds containing cytotoxic factors from whitecheek shark and their effects on enhancing the activity of natural lethal cells. According to the results, the extracted protein compounds (in a base pair with 14.5 KDa) increased the number of NK cells and enhanced their cytotoxicity at 3 mg mL^{-1} concentration. Furthermore, these bioactive proteins could increase the number and activity of T cells (i.e., NK cells) and capacitate the immune system against cancer cells. The findings demonstrated that CXCR3, NKP46, NKP44, and NKG2D could not be expressed by extracted proteins. Taken together, returning to the objectives of this study, it is now possible to state that shark cartilage is a source of biological compounds with

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stimulating properties to capacitate immune system and fight cancer cells.

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