Shark Cartilage Modulates Immune Responses in Stage III Breast Cancer Patients

Shahrokhi S,[1] Zuhair MH,[1] Mohagheghi MA,[2] Ghazanfari T,[3] Ebtekar M[1]

[1]*Department of Immunology, School of Medical Sciences, Tarbiat Modares University, Tehran, Iran*
[2]*Cancer Research Center, Cancer Institute, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran*
[3]*Department of Immunology, Faculty of Medicine, Shahed University of Medical Sciences, Tehran, Iran*

Corresponding author: *Zuhair M. Hassan, PhD*
Department of Immunology, School of Medical Sciences, Tarbiat Modares University,
P.O. Box:14115-111, Tehran, I.R. Iran
Tel: +98 21 82883565
Fax: +98 21 88006544
Email: hasan_zm@.modares.ac.ir

Abstract

Introduction: Shark cartilage has been shown to have some inhibitory effects on angiogenesis, metastasis, cell adhesion and proteolysis.

Patients and Methods: In this study, we evaluated the effect of shark cartilage on immune response in three treatment session of 3 weeks, 6 weeks and 12 weeks on stage III invasive ductal carcinoma patients (n=15) compared to patients treated with a starch placebo (n=15).

Results: The results indicated a significant increase after an initial 3 weeks treatment period in the level of IFNγ, but no significant decrease in the level of IL-4 before and after the treatment with shark cartilage. After 6 weeks, we noticed a significant increase ($P<0.05$) in the level of IFNγ, but no significant increase in the level of IL-4 was observed after the treatment with shark cartilage. After 12 weeks, a significant increase in the level of IFNγ and a significant decrease in the level of IL-4 after the treatment with shark cartilage was observed; while there was no significant difference in the levels of both IFNγ and IL-4 at 3, 6 and 12 weeks treatment in the placebo group. We also evaluated the lymphocytes proliferation in pre and post treatments with shark cartilage or a placebo. Our findings showed a significant increase in lymphocyte proliferation in the three-week treatment.

Conclusion: It is concluded that shark cartilage can stimulate immune response in a short period of time after treatment with it and modulate immune response in longer treatment duration toward Th1 cytokine pattern.

Key words: Breast cancer, IFNγ, IL-4, Shark cartilage.

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Introduction

Complementary/alternative medicine (C/AM) is of great importance, especially in the field of oncology. C/AM a great number of therapies, from acupuncture to natural medicine. The main reason for the importance of C/AM is its popularity. Recent survey data on the prevalence of the use of C/AM has provided great variations.(1–5) Shark cartilage is one of these compounds used by breast cancer patients.(2)

Oral consumption of dried powdered shark cartilage has been widely used as a natural healthy remedy for the treatment of cancer.(6, 7) Shark cartilage has been reported to inhibit tumor angiogenesis; and its extracts, when incorporated into copolymer pellets, inhibit angiogenesis in rabbit cornea, and thereby decrease the tumor size.(8) Oikawa *et al.* isolated a fraction containing 1-10 kDa proteins with the highest anti-angiogenic activity.(9) In another research, Sheu *et al,* a potent angiogenesis inhibitor U-995 was isolated, composed of two peptides, with molecular masses of 10 and 14 kDa.(10) Dupont *et al*, isolated a fraction containing 1-500 kDa molecules, called Æ-941, demonstrating anti-tumor, antiangiogenic and anti-protease activities.(11) Enhancement of CD4/CD8 in a murine tumor (which is a good prognostic indicator for cancer patients),(12, 13) an increase in the production of interleukin 12 and nitric oxide in the murine model(14) have already been reported. Also, Merly et al, evaluated the effect of shark cartilage extracts on the induction of cytokines and

Table-1. Karnofky performance status scale. Status scale definitions rating (%) criteria

Classification	Score	Condition
Able to carry on normal activity and to work; no special care needed.	100	Normal no complaints; no evidence of disease.
	90	Able to carry on normal activity; minor signs or symptoms of disease.
	80	Normal activity with effort; some signs or symptoms of disease.
Unable to work; able to live at home and care for most personal needs; varying amount of assistance needed.	70	Cares for self; unable to carry on normal activity or to do active work.
	60	Requires occasional assistance, but is able to care for most of his personal needs.
	50	Requires considerable assistance and frequent medical care.
Unable to care for self; requires equivalent of institutional or hospital care; disease may be progressing rapidly.	40	Disabled; requires special care and assistance.
	30	Severely disabled; hospital admission is indicated although death not imminent.
	20	Very sick; hospital admission necessary; active supportive treatment necessary.
	10	Moribund; fatal processes progressing rapidly.
	0	Dead

Table-2. The level of cytokines and the quality of life in the stage III breast cancer patients treated for 3 weeks with shark cartilage (n=5) and a placebo (n=5).

Subject		Before treatment ±SD	After treatment ±SD
Test group			
Cytokine assay(pg/ml):			
IFNγ		43.37±21.98	59.710±25.92*
IL-4		53.066±58.40	61.748±62.031
MTT (SI):			
-PHA	5µg/ml	1.5545±0.09	1.771±0.09
-Shark cartilage extract	150µg/ml	1.13±0.09	1.217±0.09
-Shark cartilage extract	50µg/ml	0.92 ±0.08	0.95 ±0.08
Quality of life (%)		76.00±12.05	79.00±12.86
Placebo group			
Cytokine assay(pg/ml):			
IFNγ		42.210±22.14	42.358±30.7
IL-4		36.006±30.88	42.254±33.6
MTT (SI):			
-PHA	5µg/ml	1.325±0.12	1.771±0.09
-Shark cartilage extract	150µg/ml	0.97±0.09	0.93±0.07
-Shark cartilage extract	50µg/ml	1±0.1	0.88±0.08
Quality of life (%)		75.00±2.1 5	74.00±8.74

*P<0.05; indicates a significant difference in comparison with control group as shown.
SD: Standard Deviation, SI: Stimulation index

Table- 3. The level of cytokines and the quality of life in the stage III breast cancer patients a six-week treatment with shark cartilage (n=5) and a placebo (n=5).

Subject		before treatment ±SD	after treatment ±SD
Test group			
Cytokine assay(pg/ml):			
IFNγ		39.100±20.88	78.24±23.38*
IL-4		45.320±26.91	46.320±24.86
MTT (SI):			
-PHA	5µg/ml	1.73±0.09	1.72±0.098
-Shark cartilage extract	150µg/ml	1.007±0.09	1.089±0.09
Quality of life (%)		78. 00±16.431	82.00±13.038
Placebo group			
Cytokine assay(pg/ml):			
IFNγ		39.233±28.49	39.716±26.77
IL-4		45.546±41.84	44.926±46.39
MTT (SI):			
-PHA	5µg/ml	1.018±0.09	1.50±0.09
-Shark cartilage extract	150µg/ml	0.73±0.09	1.13±0.09
Quality of life (%)		76.00 ± 15.00	71.00±21.00

*P<0.05; indicates a significant difference in comparison with the control group as shown.
SD: Standard Deviation, SI: Stimulation index

Table- 4. The level of cytokines and the quality of life in the stage III Invasive ductal carcinoma patients treated for 12 weeks with shark cartilage (n=5) and a placebo (n=5).

Subject		Before treatment ±SD	After treatment ±SD
Test group			
Cytokine assay(pg/ml):			
IFNγ		45.82±16.33	113.40±38.99*
IL-4		68.36±21.34	31.992±19.5*
MTT (SI):			
-PHA	5µg/ml	1.64±0.09	1.74±0.09
-Shark cartilage extract	150µg/ml	1.109±0.09	1.14±0.09
Quality of life (%)		82.00±8.3	84. 00±13.4
Placebo group			
Cytokine assay(pg/ml):			
IFNγ		48.16±33.25	45.0±34.65
IL-4		28.46±19.93	39.58±20.89*
MTT (SI):			
-PHA	5µg/ml	2±0.09	1.75±0.098
-Shark cartilage extract	150µg/ml	0.89±0.09	0.97±0.09
Quality of life (%)		63.00±12.1	50.00±23.6*

*P<0.05; indicates a significant difference in comparison with control group as shown.
SD: Standard Deviation, SI: Stimulation index

chemokines in human peripheral blood leukocytes. Primary leukocyte cultures were exposed to a variety of aqueous and organic extracts prepared from several commercial brands of shark cartilage. Among all of the commercial sources of shark cartilage tested, the acid extracts induced higher levels of TNFα and IFNγ at detectable levels for up to four days. Thus, it preferentially induces Th1 type inflammatory cytokines,(15) The profile of cytokines produced at any time during an immune response is largely governed by two subsets of T-helper cells, designated Th1 and Th2. Differentiation of T-helper cells into Th1 and Th2 cells is tightly controlled by the cytokines present in the local environment and the type of infection and/or immune stimulus.(16) Polarized Th1 and Th2 responses can contribute to the pathogenesis of immune-mediated diseases. Consequently, natural products and other therapeutic agents, that are able to cause shifts in the Th1/Th2 balance, could significantly influence the overall immune response.(17) The previous studies were mainly focusing on the effect of shark cartilage on the antiangiogenesis in cancer patients.(6, 7) this is because of the importance of Th1 responses in treating breast cancer patients(18) and the relationship of angiogenesis and Th1&Th2 responses.(19) Therefore, we evaluated the effect of shark cartilage usage on the immune response in pre- and post- treated stage III invasive ductal carcinoma patients. The results were compared with the placebo group treated only with starch.

Patients and Methods

Preparation of shark cartilage capsules: Neural cord cartilage was obtained from Dogfish shark, from the Persian Gulf in Iran. It was washed and scrubbed under tap water to remove the attached residual tissues and then rinsed with distilled water. The cleaned cartilage was cut into small pieces, lyophilized and then pulverized. Ten grams of the cartilage powder was extracted in 100 ml of 0.1M phosphate buffer containing 4 M guanidine HCl and a protease inhibitor cocktail (EDTA 6.25 mM, PMSF 1 mM, Benzamidine-HCl 0.25 mM, 6-Aminohexanoic acid 0.25 mM, N-Ethylmaleimide 10 mM and Iodoacetic acid 2 mM) at pH=5.8 for 48hrs with slight stirring at 2-8ºC. The extract was then centrifuged at 100,000 g for 45 minutes. The supernatant was dialyzed against PBS, centrifuged, sterilized and then lyophilized.(11, 12) The lyophilized powder of the mainly shark cartilage extract (patent No. 32185) was formulated with inert molecules from Sim1 as an herbal product from the garlic species (patent No. 32504).

Patients and the schedule of treatment: After obtaining ethic justification from the Cancer Institute of Iran for the study and informed consent of the patients, the volunteer patients then participated in this project. Thirty stage III invasive ductal carcinoma patients (aged 35-65 years), underwent the surgical procedure of radical mastectomy and hormone therapy at the Institute for Cancer in the Imam Khomeini Hospital (Tehran, Iran). The histological tumors were characterized on the basis of the following parameters by a pathologist: tumor border, appearance of the nucleus (vacuolar hyper chromatic), nuclear polymorphism, mitosis, nuclear/cytoplasm ratio, presences of nucleoli and the intensity of the

lymphoid infiltration near and within the tumor tissue. The tumors were diagnosed as invasive ductal carcinoma. The patients were randomly divided into two groups: a test and a control (placebo) group, and treated according to the following protocol:

Group 1: Shark cartilage capsules(n=5) and placebo(n=5), supplemented up to 3 times a day/ for 3 weeks:

Evaluated before the treatment,
Evaluated after the treatment,

Group 2: Shark cartilage capsules(n=5) and placebo(n=5), supplemented up to 3 times a day/ for 6 weeks:

Evaluated before the treatment,
Evaluated after the treatment,

Group 3: Shark cartilage capsules(n=5) and placebo(n=5), supplemented up to 3 times a day/ for 12 weeks:

Evaluated before the treatment,
Evaluated after the treatment,

Measurement of lymphocyte proliferation by MTT: Vein heparinized blood samples were collected and the lymphocytes were isolated by ficol hypaque (Sigma). The lymphocyte suspension was centrifuged at 2500 rpm for 10- minutes. The precipitated cells were resuspended in RPMI (Gibco BRL, Grand Island, NY, USA). Some 1×10^5 cells were poured into each well of the 96-well micro plates, and then Phytohemagglutinine (PHA, Gibco, BRL,Carlsbad, CA) and 150µg/ml of the extract were added and the mixture was incubated for 72 hours. MTT is a dye and can be taken up by mitochondria, and thus it is commonly used to determine the cellular activity and to count the number of viable cells by spectrophotometrically, measuring the mitochondrial dehydrogenase activity of viable cells.(20) Then, 20µL of MTT(3-(4, 5-Dimethyl-2-thiazolyl0-2, 5-diphenyl-2H-tetrazolium bromide, M5655,Sigma, USA) at 5 mg/mL was added into each well of the 96-well plates and incubated for 4 h in 37ºC, 5% CO2 and 90% humidity incubator. One hundred and seventy microlitres of medium with MTT was removed from every well and 100 µL of DMSO (Fisher Scientific, UK) was added to each well to extract and solubilize the formazan crystal by incubating for 20 min in 37ºC, 5% CO2 incubator. Finally, the plates were read at 570 nm wavelength using ELISA Reader (Bio-Tek Instruments, USA).(20, 21) In the present study, phytohemagglutinine (PHA) was used to identify the non specific stimulation, shark cartilage extract (for specific responses of the body towards the drug) and the cells only for a negative control. The percentage of proliferation was calculated by the following formula:

Stimulation index (SI)= OD sample− OD control/ OD control× 100

Measurement of the profile of cytokines: Peripheral blood cells were collected from the patients before and after the treatments with shark cartilage capsules and a placebo. The lymphocytes were isolated by ficol hypaque and the lymphocyte suspension was centrifuged at 2500 rpm for 10 minutes. The precipitated cells were resuspended in RPMI containing 10% fetal calf serum. 2×105 cells were poured into each well of the 96-well micro plates, and then PHA was added. The resulting mixture was incubated for 72 hours at 37°C and 5% CO2. Then the supernatants were collected. An ELISA Kit (BMS228_02) and (BMS225/2, Bender Med Systems GmbH Campus Vienna Biocenter, Austria,) was used to measure IFNγ, IL-4. Briefly, after washing the wells with a buffer, the standard samples were added to each well and then biotin conjugates were added. The resulting mixtures were incubated for 2 hours. The micro plates were washed three times with washing buffer and Stereptoavidin- HRP was added. The plates were incubated for 1 hour at 37ºC, and then washed by a washing buffer. The TMB substrate solution was dispensed for 15 minutes, and then a stop solution was added. ELISA reader (450 nm filter) was used to read the results.

The quality of life of the patients treated with shark cartilage capsule: The breast cancer patients were divided into two groups and treated according to the protocol mentioned above. The physical and performance status of the cancer patients were assessed by the Karnofsky Performance Scale Index(22, 23) as a quality of life score which ranged from 0-100 (Table-1). The Karnofsky-Index allows patients to be classified as to their functional impairment. It is also used to compare the effectiveness of different therapies and to assess the prognosis in individual patients. The lower the Karnofsky score, the worse the survival for most serious illnesses.

Statistical analysis: To determine the statistical significance of the data, pair T-test and independent T test were used. In all the analyses, statistical significance was claimed at 5% level ($P<0.05$).

Results

The effect of a three-week treatment of shark cartilage capsules on immune response: In order to assess the effect of shark cartilage on immune response, the 10 stage III breast cancer patients (A and B) were divided into two groups and treated according the protocol of the three-week treatment already mentioned. The results of the MTT assay indicated that shark cartilage treatment had no significant effect on cell proliferation compared to before the treatment test and the control groups, while the PHA showed a significant increase compared to its score before the treatment. The results of ELISA showed a significant increase of IFNγ in the test group ($P<0.05$), but no significant decrease was observed for IL-4($P>0.05$). Also, no significant change was observed for the placebo group after the treatment with shark cartilage (Table-2).

The effect of a six-week treatment of shark cartilage on immune response: The results of lymphocyte proliferation in a six- week treatment of patients indicated that the shark cartilage in the test group showed no significant difference regarding this test at the dose of 150µg/ml, compared to its score before the treatment of the test group. However, the results indicated a significant increase of IFNγ ($P<0.05$), but no significant decrease was observed for IL-4 ($P>0.05$) after the treatment (Table-3). The placebo group also showed no significant difference in all mentioned parameters used before and after the treatment.

The effect of a twelve-week treatment of shark cartilage on immune response: To evaluate the effect of shark cartilage on immune response, the 10 stage III breast cancer patients (A and B) were divided into two groups and treated according to the protocol of 12 weeks of treatment. The results of lymphocyte proliferation indicated that there was no significant difference in terms of the effect of shark cartilage at a dose of 150µg/ml on the immune response of the pre- and post- treated test groups. However the results of ELISA showed a significant increase for IFNγ ($P<0.05$) and a significant decrease IL-4 (Table-4) in the patients treated with shark cartilage. The placebo group showed a significant increase in the level of IL-4 compared to with the level of IL-4 in the pre- and post- treated patients with shark cartilage.

The quality of life of the patients treated with shark cartilage-Sim1: Before and after the treatment, the quality of life of the breast cancer patients in the test and control groups was scored by Karnofsky scale. A significant ($P>0.05$) decrease in the quality of health was seen in the placebo group after 12 weeks (Tables 2, 3, 4).

Discussion

Complementary /alternative medicine, which includes therapies such as acupuncture and herbal medicine, has been defined as those forms of health care provisions that usually lie outside the official health sector.(24) Several studies have investigated the application of C/AM use for cancer patients. The prevalence of the uses of C/AM is relatively high.(25-28) The relative lack of success with individual therapies shows that the direction should emphasize a combination of strategies. It is notable that most of the agents, showing activity in murine and human cancer, have complementary immunopharmacologies, so that the combined use of vaccine, adjuvant, cyclophosphamid and interleukins should be compatible. Many strategies seem to be possible and logical to achieve tumor specific immune responses without sever toxicity and great expense.(14, 29, 30) One of these compounds is shark cartilage which is widely used by cancer patients. Since the discovery that sharks rarely develop cancer,(6) some progress has been made in identifying the various unusual compounds present in shark cartilage. It is clearly known that there are some antiangiogenic compounds in shark cartilage that make it quite resistant to tumors. It has several mechanisms such as antiangiogenesis, immunostimulation, anti- inflammation and many other protective mechanisms.(6) Interest in shark cartilage stems from early research reported in two compelling studies. The first study involved glycoproteins isolated from hammerhead sharks. These glycoproteins extended life in leukemic mice.(31) The second study implanted shark cartilage pellets intraocularly in rabbits, which inhibited tumor angiogenesis.(32) In vitro and pharmacokinetic studies have indicated that the mechanism of the action of shark cartilage is through the prevention of neovascularization and the subsequent inhibition of cell proliferation.(33, 34, 35, 36) Evidence has also suggested that shark cartilage may protect against mutagenesis and DNA lesions.(37, 38) Cytotoxic activity of shark peripheral blood leukocytes has also been reported. To date, researches on shark cartilage have been focused mostly on its anti-angiogenic effects.(7-11) Other identified effects of shark cartilage include: an inhibitory effect on metastasis, on cell adhesion,

on some proteases such as Matrix Metalloproteinase, on *in vitro* growth of cancer cell lines and on DTH response.(12)

Due to the direct evidences of the immunostimulatory activity of shark cartilage(24, 25, 26) in murine models, and the importance of the acquisition of an immune reaction, which plays a critical role in the prevention of tumor progression, shifting towards the Th1 profile on the basis of the predominance of IFNγ and decreasing interleukin 4 are important in assuming ongoing responses of T cells against the tumor burden.(18) Because of this background knowledge, we investigated the immunological effect of shark cartilage on human breast cancer as a clinical trial during different treatment durations.

The results indicated that the level of IFNγ and IL-4 reached a stable state after three weeks. Then, a significant increase in the level of IFNγ as well as a decrease in the level of IL-4 occurred in the patients treated fot 12 weeks. IFNγ and IL-4 are two important factors for controlling tumor progression.(8) The stimulation of the immune system was observed with a significant production of IFNγ and lymphocyte proliferation in PHA test. The best response was seen in the patients supplemented for 12 weeks with shark cartilage capsules. Thus, we saw a shift of cytokine response to type 1 which is a very important protective and defensive mechanism against tumor. During short-term treatment protocols, at first, we noticed immunostimulation. Then, as the therapy progressed, immunomodulation was observed. Therefore, we recommend long-term treatment for these cancer patients, but further studies are needed to confirm this idea. We noticed a significant increase in the level of IL-4 and a stable level of IFNγ in the patients treated with starch as placebo indicating a continual shifting toward T helper 2. Our results agree with those of the Merly et al. study,(15) that the most well-characterized cytokine response of human peripheral blood to shark cartilage stimulation is a proinflammatory cytokines induction. Thus, the enhanced production of, TNFα, IFNγ, and IL-1β suggests that shark cartilage preferentially induces a Th1 type cytokine response. Shark cartilage did not induce a significant decrease in the level of IL-4, thus it only preferentially stimulates a Th1 type response but it appears to indirectly inhibit the development of a Th2 response through the action of IFNγ, which is an inhibitor of Th2 cell population expansion.(30, 39)

The effect of different extracts of shark cartilage on the cytokine response indicated that the most cytokine inducing activity was associated with the acid extracts of shark cartilage. Acid extracts simulate the acidic environment of the stomach. For a dietary supplement to be biologically effective at the time of absorption, it must be acid resistant. Considering that the acid extract of shark cartilage is the most effective inducer of a cytokine response, the acidity of the stomach may very well play a role in the in vivo release of the active component(s) from crude cartilage preparations taken orally. When considering in vivo conditions, the presence of microbial enzymes must also be taken into consideration. This could be a factor in determining the potential effectiveness of shark cartilage as a dietary supplement.(15)

Thus, if through intestinal absorption the active component(s) in shark cartilage are to reach systemic circulation and/or target sites in the body, immune regulation could be significantly influenced.(15) This is in agreement with our results, owing to a similarity in its acidic preparation condition.

The responses of T lymphocytes to PHA in the three week treatment group: in the placebo patients, we noticed a significant increase in the lymphocyte proliferation in the three and six week groups and significant decrease in the twelve week treatment group against PHA. In fact, in cancer patients, low lymphocyte proliferation is mostly correlated with the progression of tumor(28) responses. No specific responses were noticed in the T cell proliferation towards an in vitro culture with shark cartilage which shows that it has no nonspecific proliferate activity on lymphocytes. But, in the study of Merly et al, it was concluded that an active component in shark cartilage behaves like a mitogen stimulating leukocytes like PHA. This difference may be related to the condition of cultivation because for an efficient stimulation of the immune system to tumor, we do not need nonspecific proliferation which may aggravate the tumor.

Our results agree with the previous immunological studies on shark cartilage, in terms of enhancement of CD4/CD8 in murine tumor (which is a good prognostic indicator for cancer patients),(25, 29) the increased production of interleukin 12 and nitric oxide in murine and the human model (24, 40, 41) and the shifting of responses towards Th1 which can prevent tumor growth and metastasis.

In a previous study (Loprinzi et al) it was indicated that shark cartilage was not effective in advanced breast or colorectal carcinoma patients.(42) There was no difference in overall survival and the quality of life between patients receiving standard care plus

a shark cartilage product versus standard care plus a placebo. Probably in late stages of cancer the microenvironment of the carcinous tissue changes to Th2. In our study, when we gave shark cartilage to stage III breast cancer patients, we notice a significant increase in their IFN levels, which indicated reverse a microenvironment from Th2 to Th1.

It is important to note that shark cartilage had no toxic effect on the normal cells and its anti-angiogenic activity appears to only be applied to new vessels. Therefore, it can be administered for a longer period to achieve good results. It can be concluded that shark cartilage can stimulate immune response during short-term treatment and modulate this response in long-term treatment.

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