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Effects of Shark Cartilage on Heterotransplanted Oral Squamous Cell Carcinoma Induced in Nude Mice

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Objectives The use of shark cartilage as a supplementary treatment has a long yet unresolved history in the realm of complementary-alternative medicine. This study aimed to investigate the impact of concentrated and purified extracts from Persian Gulf shark cartilage (PGSC) on oral cavity squamous cell carcinoma (SCC) (specifically, the KB cell line) induced in an animal model.
Methods Ectopic tumors of oral cavity SCC were induced in eight nude mice through the heterotransplantation of the KB cell line. Once the tumor volume reached 100 mm3, the mice were randomly divided into two groups: treatment and control. The treatment group received shark cartilage at a dosage of 50 mg/kg body weight, while the control group received a phosphate buffer. The drug was administered daily for four days via the intraperitoneal route. Following this, the drug administration was halted for a period of five days before resuming (as per the NCI protocol). After 54 days, the animals were sacrificed, and their tumors were sent for immunohistochemical evaluation using Ki-67 and CD34 markers.
Results The findings revealed a significant reduction in intratumoral blood vessels in the treatment group compared to the control group (P-value = 0.001). While there was a decrease in both the size of the tumor and the proliferation of tumor cells, this reduction was not statistically significant. The average proliferation index was 13.33% for the treatment group and 33.33% for the control group.
Conclusion Significant decrease in intra-tumoral vascularity can control tumor spreading and metastasis, potentially playing an important role in cancer management of oral cavity SCC.
Keywords Squamous cell carcinoma; Shark cartilage; CD34; Ki-67; Oral cancer

Introduction

Squamous cell carcinoma (SCC) is the most common cancer of the oral cavity. [1, 2] Each year, over 30,000 new cases of mouth and oropharynx cancers are reported in the United States. This disease is also responsible for about 7,000 deaths annually. [3] The treatment of mouth and oropharynx cancers not only imposes a significant financial burden on the healthcare system, but also leads to numerous restrictions and complications for the patients. These complications can have severe consequences, including disability and mental illness. Therefore, the timely treatment of primary lesions and controlling their recurrence is crucial in preventing cancer progression and enhancing patient survival. Surgery is the primary treatment method for SCC, often used in conjunction with other treatments, such as radiotherapy and chemotherapy. [4, 5] Given the physical and mental impairments resulting from ablative surgeries, which are often the most definitive treatment for many cancers, researchers are exploring alternative treatment methods to reduce these side effects. Such methods include immunotherapy, photodynamic therapy, gene therapy, and alternative therapy. [6-12]

Complementary and alternative medicine (CAM) encompasses a wide range of practices, materials, and treatments, such as homeopathy, energy healing, acupuncture, herbal remedies, and biologically based treatments including shark cartilage, aimed at enhancing the healing effects of drugs. Unlike mammals, the shark's skeleton is primarily made of cartilage, which could explain their extremely low incidence of neoplasms. While the scientific evidence surrounding CAM in general, and shark cartilage preparations specifically, remains controversial and inconclusive, several studies have suggested that shark cartilage contains substances that inhibit the formation of new blood vessels into solid tumors, thereby limiting tumor growth. [13-15] Researchers at Tarbiat Modares University (Tehran, Iran) have investigated the inhibitory effects of Persian Gulf shark cartilage (PGSC) on breast cancer and found that it can increase patient survival. [16] Given that no animal or human studies have been conducted on the effects of shark cartilage on SCC of the oral cavity, this study aimed to investigate the impact of condensed and purified extracts from PGSC on the epithelial cells of oral cavity cancer (specifically, the KB cell line) introduced into an animal model.

Methods and Materials

This study utilized the following materials: 0.25% trypsin-EDTA (Gibco, USA); fetal calf serum (Gibco, USA); high-glucose DMEM medium (Gibco, USA); 10-mL sterile serological pipette (TPP); 50- and 250-mL cell culture flasks (BD Bioscience, USA); 15-mL sterile

centrifuge tubes (Jet Biofil, China); 0.22-µm pore size syringe filter (Jet Biofil, China); shark cartilage; 96-well microtitration plate (BD Bioscience, USA); PBS tablets (Gibco, USA); 35-mm petri dishes (Jet Biofil, China); the KB cell line (Cell Bank, Pasteur Institute of Iran); athymic nude mice (Mecomex); and CD-34 and Ki-67 immunohistochemical (IHC) staining kit (Dako, Denmark).

Cell culture

To prepare and increase the number of cells, the KB cell line underwent a three-step process. The cell line was cultured in DMEM containing 4500 mg/L of glucose and 10% fetal bovine serum (FBS), and the flask was incubated at 37°C with 5% CO2. The culture medium was replaced every two days. When cell confluency reached approximately 85%, the medium was removed, and the cells were washed with phosphate buffered saline (PBS). Then, 1 mL of trypsin (0.25%) containing 1 mM EDTA per 25 cm2 flask was added, and the cells were incubated for three minutes. Once the cells detached from the flask, cell culture medium containing 10% FBS was added. The cell suspension was then transferred to a 15-mL centrifuge tube and centrifuged at 200 g for seven minutes. The supernatant was discarded, and the cell pellet was re-suspended in Complete Cell Culture Medium. The cells were then counted using a Neubauer chamber under a light microscope. Subsequently, 450,000 cells per 25 cm2 area of the flask were added to new flasks. All procedures were performed within a Class II Biosafety Cabinet.

Xenograft study

To establish the xenograft model of the KB cell line, 10 female athymic nude (Nu/Nu) C56BL/6 mice, aged six weeks, were selected. These mice were housed in separate ventilated cages at an animal facility. The average temperature inside the cages was maintained at 21°C, with an average relative humidity of 65%. All animals were kept in sterilized conditions and were provided with standard laboratory chow and water ad libitum, specifically for immunodeficient mice. This study adhered to the 3R (reduction/refinement/replacement) principles for working with laboratory animals, in compliance with all institutional and national guidelines for the care and use of such animals.

Given the potential for tumor formation failure, which is a common and established concept in cancer studies, a pilot study was initially conducted to ensure histopathological similarity between the developed tumors and the inoculated cell line, as well as SCC. The cell line was procured from the Pasteur Institute of Iran. Following expansion and preparation, 200 µL of serum-free culture medium containing 5×10^6 cells was inoculated into each flank of two mice using a 27-gauge insulin syringe. This procedure was carried out within a Class II Biosafety Cabinet following physical subjugation.

The large and small diameters of the formed tumors were measured on average every 3-4 days using a digital caliper, and the tumor volume was calculated using the following equation [17]:

Tumor volume = Large diameter × (Small diameter)^2 × 0.52

Once the tumor volume reached approximately 100 mm^3, eight mice with well-formed tumors were randomly divided into two groups: a shark cartilage treatment group and a control group. The treatment group was administered shark cartilage at a dosage of 50 mg/kg body weight. The drug was injected intraperitoneally daily for four days, followed by a five-day pause in drug administration, after which the medication was resumed, based on the National Cancer Institute (NCI) protocol. The control mice were treated with equal volumes of PBS. The total treatment period was set at 54 days, divided into six periods of 4-5 days. On day 54, the animals were sacrificed by carbon dioxide inhalation, and the tumors were stabilized in a 10% buffered formalin solution and sent to the pathology laboratory for further analysis.

Shark cartilage preparation

The cartilage from the spinal cord of a Persian Gulf shark was cleaned and washed with water to eliminate any residual tissues. The cleaned cartilage was then cut into small pieces and lyophilized to create a powdered form. Initially, 38.2 g of guanidinium hydrochloride was dissolved in 100 mL of 0.1 M acetate buffer solution. After adjusting the pH to 5.8, 1 mL of PMSF (1 mM) was added to this solution. For every 10 mL of guanidinium hydrochloride acetate, 1 g of shark cartilage powder was added. This mixture was stirred for 48 hours at 4°C using a magnet. Following a 30-minute centrifuge of the solution at 3000 rpm, the supernatant was dialyzed for 24 hours in PBS using a dialysis bag. The contents of the dialysis bags were finally stored at -20°C. The presence of the intended protein was confirmed using sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 1).

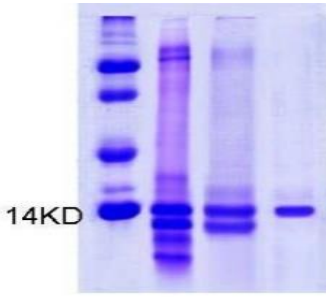

Figure 1: Intended Protein of the shark cartilage separated by SDS-PAGE

Histopathology

To verify the nature of the xenograft neoplasia, the

stabilized samples underwent various stages of dehydration and were then prepared into paraffin blocks. Sections of 3 µm were prepared from these paraffin blocks and subsequently stained using the H&E method. These sections were then evaluated by an experienced oral and maxillofacial pathologist. Moreover, to assess the effect of shark cartilage on cancer cell proliferation and tumor angiogenesis, the samples were examined using the IHC technique. Paraffin blocks were placed in ice water for 30 minutes, and 4 µm-thick slices were prepared using a microtome. After a brief drying period of 15 minutes, the sections were fixed at 37°C on slides. The cross sections were then deparaffinized and dehydrated with varying concentrations of ethanol.

To examine proliferation and angiogenesis, the sections were stained with antibodies against Ki-67 (M7240, Dako, Denmark; 1:75) and CD34 (M7165, Dako, Denmark; 1:50), following the manufacturer's kit instructions. Briefly, antigen reduction was performed using heat and citrate buffer treatment for 10 minutes. The sections were then exposed to 5% hydrogen peroxide and absolute methanol and finally washed with distilled water. The samples were incubated with the primary antibody for 10 minutes. In the next step, a secondary antibody was added for 10 minutes, and then, the sections were washed with PBS. The samples were exposed to diaminobenzidine as a chromogenic substrate for 10 minutes. To assess the impact of shark cartilage on the proliferation of cancer cells, a measure known as the proliferation index was utilized. Sections stained with an antibody against Ki-67 were examined at 400x magnification. At this magnification, a minimum of 1000 cells were counted across 10 fields. The proliferation index was then calculated by dividing the number of immunoreactive nucleated cells by the total cell count and multiplying the result by 100.

To evaluate angiogenesis, a measure known as microvessel density (MVD) was used. Sections stained with an anti-CD34 antibody were initially evaluated at 40x magnification to identify three to six areas with the highest vascular density (hot spots). At 400x magnification, endothelial cells with a dark nucleus were counted. During the counting process, the presence of a lumen was not considered a prerequisite for identifying the vessel structure, and necrotic cells were carefully excluded from the count of immunoreactive cells. The average count was recorded for each slide. Ki-67 and CD34 were used as positive controls for intestinal tissue and bone marrow, respectively. Samples stained only with the secondary antibody (without the primary antibody) were considered negative controls for both markers. It is important to note that the pathologist was not aware of the origin of the samples.

Data analysis method

Data was presented as mean ± standard error (SE). Student's t-test was used to compare the average of the groups in terms of MVD and proliferation index. The significance level was set at 0.05. Statistical analysis was performed using the Graph Pad Prism 6.

Ethical considerations

This study was conducted in accordance with the ethical principles of working with laboratory animals, adhering to the 3Rs (reduction, refinement, and replacement) principles. It was approved by the ethics committee at Shahid Beheshti university of Medical Sciences (no.154) and the research committee (no.339).

Results

In this study, 10 athymic nude mice, each approximately six weeks old, were heterotransplanted with the KB cell line. A pilot study was conducted on two samples. In one mouse, tumor formation began on day five, and the mouse was euthanized on day 48. The developed tumor, located on one flank, was extracted and divided into five pieces (Figure 2). One of these pieces was sent to a pathology laboratory for SCC diagnosis, while the remaining four pieces were separately transplanted into four other mice. Microscopic examination of H&E-stained lesions confirmed the squamous nature of the lesion, indicated by changes in the differentiation pattern (ranging from poorly differentiated to undifferentiated). The second mouse, which exhibited tumor formation in both flanks from day 13, was euthanized on day 53. Similar to the first mouse, the tumor was used for the histopathological study and serial transplantation in other mice. The squamous nature of the lesion was also demonstrated by changes in the differentiation pattern (from poorly differentiated to undifferentiated).

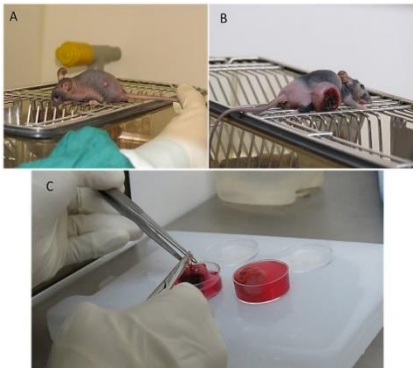

Figure 2:
A: Pilot study on mice showed evidence of tumor formation at day 5.
B: Overview of the tumor at day 48.
C: Tumor Removal and division for serial transplantation.

The bilateral injections were administered in accordance

with the proposed NCI protocol for xenograft studies to prevent any interference in the study due to a prolonged delay in tumor formation. Of the eight mice, two were excluded from the study due to unintentional death, and autopsy was not possible due to autolysis (T2 mouse on day 48 and C3 mouse on day 22). The total test period was 54 days, consisting of four days of injection and a five-day interruption. However, mouse C4 (control) was sacrificed on day 47 (one week earlier) due to distress. In each of the remaining six mice, tumors formed bilaterally, but only samples from one side were randomly sent for microscopic examination in the pathology laboratory. The growth curve for the mean tumor volume of both sides is illustrated in Figure 3, excluding the two unsuccessful mice. Tumors were dispatched to the laboratory from the following locations: the left flank of the three mice in the treatment group (T 1-3-4), the left flank of the C2 mouse, and the right flank of the C1 and C4 mice. It is important to note that the pathologist was not aware of the group assignments of the tumor samples, ensuring an unbiased examination.

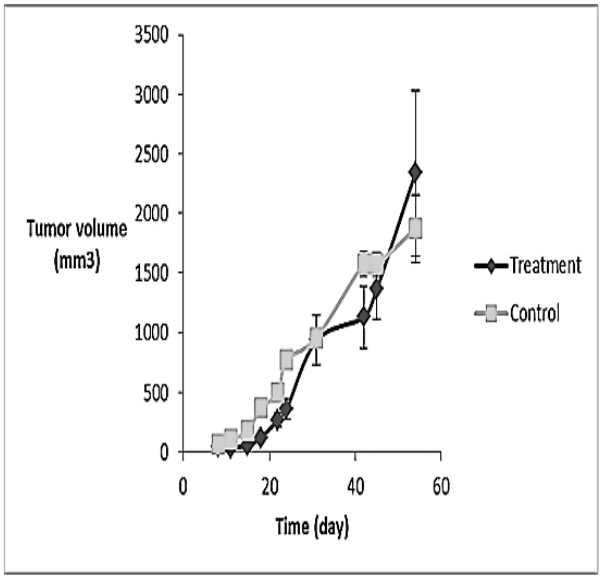

Figure 3: Kinetic curve of tumor growth

Evaluation of proliferation index

The expression of the Ki-67 marker showed no significant difference between the treatment and control groups (13.33±7.13 vs. 33.33±6.9). The calculated P-value was 0.1138, which is not considered significant (P>0.05) (Figures 4a & 5b).

Evaluation of MVD

The MVD in the tumors of the treatment group was significantly lower than that in the control group (2±1.15 vs. 13±0.58) (P=0.001) (Figures 4b & 6d).

Other histopathological findings

In addition to the findings discussed earlier, other observations were also made. In all three samples from the treatment group, the differentiation pattern of SCC changed to undifferentiated. In contrast, all three samples from the control group were reported to be poorly differentiated SCC. All tumors in the treatment group had a capsule, while tumors in the control group had an incomplete capsule or no capsule (Figure 6c). In the treatment group, tumor cells were localized closer to the capsule at the periphery, and extensive necrosis was observed at the center of the tumor (Figures 5c & 6a). However, in the control group, necrosis was scattered throughout the entire lesion in an island form, and unlike the treatment group (with broad and generalized necrosis at the center), necrosis was also diffused. Mitosis and other specifications are shown in Table 1. The average mitosis was 8±2 in the control group and 3.6±0.46 in the treatment group; this difference was not statistically significant assuming a P-value <0.05 (P=0.0978). However, assuming a P-value <0.1, this reduction was significant. In the IHC staining for CD34, even staining was observed in tumors of the control group, while in the treatment group, focal staining was observed.

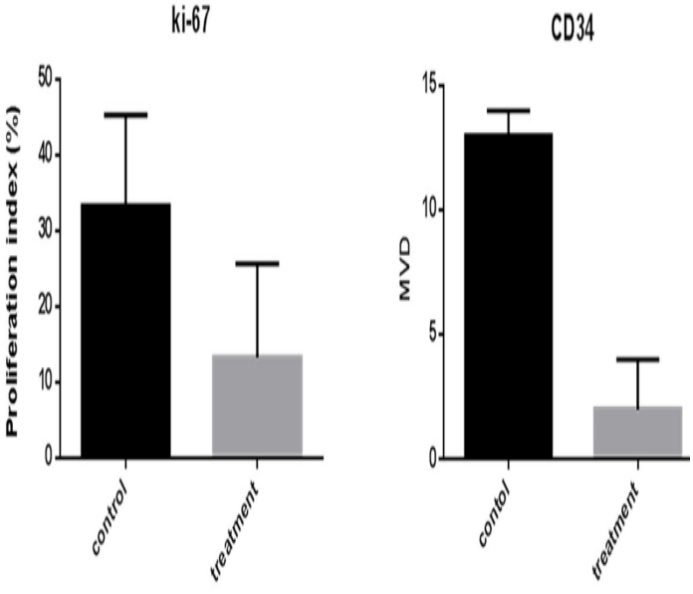

Figure 4:
A: diagram of proliferation index
B: diagram of micro-vessel density

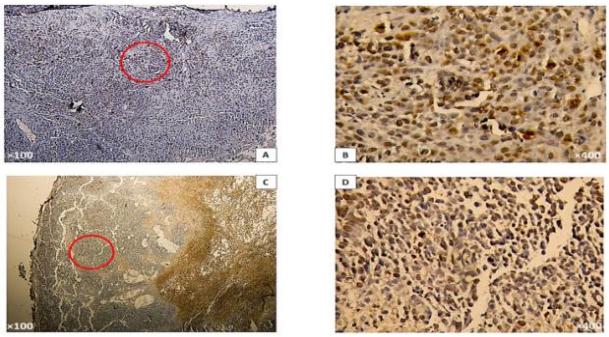

Figure 5:
Positive Ki-67 immunostaining of C4 RF (A, B) and T1 LF (C, D) that show proliferation rate in control group higher than treatment group (A, ×100) (B, ×400) (C, ×100) (D, ×400)

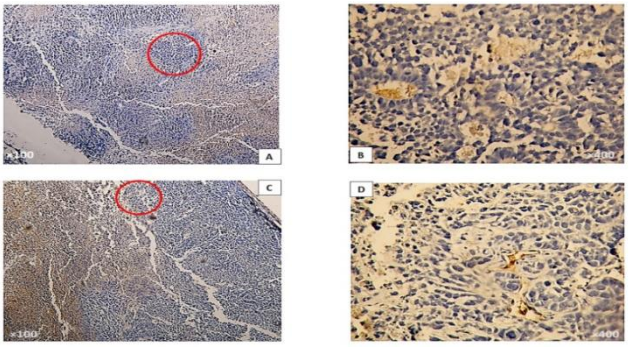

Figure 6:
Positive CD34 immunostaining of C4 RF (A, B) and T1 LF (C, D) that show MVD in control group higher than treatment group (A, ×100) (B, ×400) (C, ×100) (D, ×400).

Table 1: Summary of observations and pathology reports

Tumor	Differentiation of tumor	Ki-67	CD34	Mitosis	Capsule
T1LF	Undifferentiated	10 %	0	3.25	Yes
T3LF	Undifferentiated	3 %	2	4.5	Yes
T4LF	Undifferentiated	27 %	4	3	Yes
C1RF	Poorly differentiated	37 %	14	4	Partially
C2LF	Poorly differentiated	43 %	13	10	Partially
C4RF	Poorly differentiated	20 %	12	10	Partially

Discussion

Ever since it was discovered that sharks seldom develop cancer, a multitude of studies and experiments have been conducted to pinpoint the specific responsible compounds in shark cartilage. It is now widely acknowledged that shark cartilage contains potent anti-angiogenesis compounds that inhibit them from contracting cancer. [15, 18, 19] Following Dr. J. Folkman's research on the crucial role of vascularization in tumor growth and metastasis, the scientific community turned its attention to the potential anti-tumorigenic properties of shark cartilage. Dr. Folkman's theory, proposed in 1972, emphasized that an increase in the tumor cell population necessitates a corresponding expansion of the tumor's capillary bed. [20-22] This theory has opened up new avenues in cancer treatment, leading to the development and investigation of a broad spectrum of both natural and synthetic materials with anti-angiogenesis properties.

Cartilages, particularly shark cartilage, are among the first natural materials utilized for their anti-angiogenic properties. However, what primarily sparked the scientists' interest in cartilage is the avascular nature of this tissue, which suggests the presence of anti-angiogenic compounds. Further clinical evidence bolstering this hypothesis, which has intrigued scientists, encompasses the non-aggression of osteogenic sarcoma towards adjacent cartilage tissue, the rare occurrence of spinal column cartilage involvement following breast cancer metastasis to the spine, and the observation that chondrosarcoma possesses the least number of vessels among solid tumors. [21] Initially, calf cartilage was employed for its anti-angiogenic properties. However, the high ratio of cartilage to body weight in sharks, coupled with their low incidence of cancer, led researchers to favor shark cartilage. Beyond cancer, several other diseases have been identified as angiogenesis-dependent, including arthritis, psoriasis, and diabetic retinopathy. [21, 23, 24]

The effects of AE-941 (Neovastat, a shark cartilage) have been studied in animal models for both ulcerative colitis and rheumatoid arthritis. In both instances, the results demonstrated the efficacy of this product in managing the progression of these diseases. [25, 26] The impact of shark cartilage and its extracted constituents on angiogenesis-dependent diseases, notably cancer, has been a subject of debate. Since the topic was introduced, a variety of shark cartilage components have been examined. The discrepancies in the findings could be attributed to differences in the processed components or even the species of shark. Studies commonly use two commercial components: U-995, derived from the Prionace glauca species, and AE-941, derived from an unidentified species. AE-941, also known as Neovastat, is typically used as the benchmark material in most studies. Besides its anti-

angiogenic property (which was validated using the CAM model), this compound exhibits a potent inhibitory effect on collagenase activity. [21] Consequently, in 1998, the NCI sanctioned the use of this material for phase III multi-center clinical trials. This was done in partnership with the manufacturer of these products (Aeterna Laboratories, Quebec, Canada). [21] However, the results from human studies are inconsistent and correspond with in vitro and in vivo animal experiments.

In a review study conducted by Raimundo Pajón González in 2001, several factors were identified that could account for the inconsistent results across different tests. One such factor is the selection of end-stage cancer patients with a poor prognosis for clinical trials. In these patients, not just shark cartilage, but even fundamental treatments, such as chemotherapy and surgery, often fail to yield successful outcomes. Another factor contributing to the inconsistency is the route of drug administration. In many studies, the drug was administered orally, but there is a belief that the primary ingredient of shark cartilage, which is protein, is degraded during digestion. Interestingly, despite concerns about the vulnerability of shark cartilage extract in the digestive system, some studies have reported positive findings with oral administration of shark cartilage, supporting its effectiveness. [27-29] Despite the ongoing debate and conflicting results, the use of complementary therapies, including shark cartilage, in cancer patients has been on the rise. In fact, some studies have reported that the application of CAM ranges between 48% and 88% among these patients. [30, 31]

The present study builds upon the research led by Hassan et al. at Tarbiat Modares University (Tehran, Iran). They successfully extracted and purified the most potent fraction with an immune-stimulating effect from shark cartilage. Following a series of in vitro and in vivo experiments, they demonstrated the effectiveness of this fraction in treating patients with stage III breast cancer. [16, 18, 32] The key distinction between this study and the experiments conducted by Hassan et al. lies in the source of the tumor cells and the length of drug administration. Their experiments utilized a murine cell line or allograft tumors in mice. In contrast, the current study employed xenograft tumors in nude mice.

The nude, or athymic, mouse is a type of laboratory mouse that lacks a thymus due to a genetic mutation, resulting in a negligible number of T cells and an absence of an immune system. Due to the presence of two copies of the "nu" gene, the primary observable characteristic in this mouse is its hairlessness, which is why it is referred to as a "nude" mouse. This mutation was first identified in mice by Grist in 1962 at a virology laboratory in Glasgow. Nude mice are highly valued in research because they can accept various types of transplanted tissues or tumors. As such, they are frequently used in studies of the immune system, cancer treatment drugs, solid tumor evaluation, leukemia, AIDS, and other immune deficiencies. Owing to the absence of functional and mature T cells, nude mice are incapable of rejecting both allografts and xenografts. These mice typically live for six months to one year, but their lifespan can be extended to 18 months if they are kept in germ-free, controlled conditions and given antibiotic injections. [33]

In the research conducted by Hassan et al. in 2005, it was found that the tumor size in mouse adenocarcinoma was significantly reduced when treated with 14- and 15-kDa fractions of shark cartilage. Similarly, a study by Falardeau showed that the oral administration of Neovastat led to a substantial decrease in the size of breast cancer (DA3) tumors in mice. [18, 34] In the present study, the used dosage and protein fraction were similar to those in the study by Hassan et al. in 2005. However, as indicated in Figure 3, there was no significant difference in tumor size reduction between the treatment and control groups. One possible explanation for this minimal decrease could be the absence of T lymphocytes in nude mice. The infiltration of lymphocytes into the tumor is one of the ways the host's immune system can control tumor progression. It has been also established that lymphocytic infiltration is reduced in growing tumors. [18, 35, 36]

Much of the research on shark cartilage has focused on its anti-angiogenic properties. A notable study by Oikawa et al. in 1990 reported that protein fractions ranging from 1 to 10 kDa, extracted from a Japanese shark, exhibited the highest anti-angiogenic activity. [37] In a separate study by Sheu et al. in 1998, a potent anti-angiogenic compound, known as U-995, was identified. This compound is composed of two proteins, one weighing 10 kDa and the other 14 kDa. [38] The AE-941 compound, also known as Neovastat, was identified as a result of research conducted by Dupont. [14] Moreover, the study by Liang et al. in 2000 isolated a fraction that exhibited the most potent inhibitory effect on the growth of endothelial cells. [39] So far, numerous studies have isolated various compounds, each claiming to possess the most potent anti-angiogenic and anti-tumorigenic effects. However, aside from AE-941 and U995, which are the most prominent and commonly used substances, other compounds have not achieved widespread recognition. Most of these studies rely on the rabbit corneal assay (CAM) and endothelial cell cultures for in vivo and in vitro evaluations of angiogenesis, respectively.

In the 2005 study by Hassan et al., the specified fraction was exposed to human brain microvascular endothelial cells (HBMEC) in an in vitro setting. The results demonstrated the prevention of neo-angiogenesis in the test group when compared to the control group. In the

present study, we chose to evaluate the effect of the targeted fraction using in vivo mouse models and IHC staining for CD34 markers for a more precise assessment. As no previous research has investigated the anti-angiogenic effects of shark cartilage using vascular markers, such as CD34, CD31, or von Willebrand factor (which are considered the gold standards in angiogenesis studies) [40, 41], our research is unique and noteworthy. CD34, also known as the human hematopoietic progenitor cell antigen, is a 110-kDa protein. It is expressed by embryonic cells of the hematopoietic system, including myelogenous elements, lymphoid cells, and endothelial cells. [42, 43] CD34 is a potent marker for vascular differentiation in soft tissue tumors. After performing IHC staining for the CD34 antigen, the results are quantified using a measure known as MVD. [42, 44-46] This study revealed a significant decrease in the MVD of tumors treated with shark cartilage. Not only was there a difference in the quantity of MVD, but the quality of staining also varied between the two groups. The staining of tumors in the control group was homogeneous, while in the treatment group, it was focal, indicating that neovascularization was limited to specific areas of the tumor. Another observation was the absence of staining in the tumor cells of both groups, which could be attributed to a decrease in tumor cell differentiation.

Ki-67 is a non-histone nuclear protein that is utilized as a marker for assessing cell proliferation. It is present during all active phases of the cell cycle (G1, S, G2, and mitosis), but is absent from resting cells (G0). [42, 45, 46] In this study, we examined both macroscopic and microscopic tumor tissue proliferation, as represented by tumor growth kinetics curves. Our results indicated a marginal decrease in tumor tissue proliferation in the treatment group compared to the control group. However, this difference was not statistically significant. Previous studies have primarily focused on the anti-angiogenic properties of shark cartilage. In contrast, our study aimed to evaluate the anti-tumorigenic effects of this compound in a more objective manner by using the Ki-67 marker and assessing histopathological changes

Conclusion

Significant decrease in intra-tumoral vascularity can control tumor spreading and metastasis, potentially playing an important role in cancer management of oral cavity SCC.

Acknowledgement

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Conflict of Interest

No Conflict of Interest Declared ■

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