



AMERICAN UNIVERSITY OF BEIRUT

THE EFFECT OF BOVINE CARTILAGE ON SURVIVAL OF  
B16F10 MELANOMA AND ON MOUSE MONONUCLEAR CELLS  
IN VIVO AND IN VITRO

By

ARAX ARTIN TANELIAN

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Submitted in partial fulfillment of the requirements  
for the degree of Master of Science  
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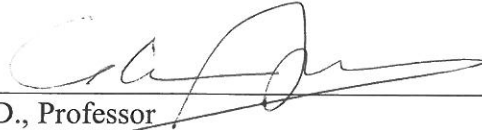
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*In the loving memory of  
Antranik Harutunian*

# AN ABSTRACT OF THE THESIS OF

Arax Artin Tanelian

for Master of Science

Major: Microbiology and Immunology

Title: The Effect of Bovine Cartilage on Survival of B16F10 Melanoma and on Mouse Mononuclear Cells *in vivo* and *in vitro*

Background and Aims: Promising results were obtained when bovine cartilage was used to treat several malignancies. However, only a few *in vitro* and *in vivo* studies were conducted to assess its mechanism of action. Additionally, no research was done to study its effect on healthy non-cancerous cells. The aim of this study was to investigate some of the proposed mechanisms of action of bovine cartilage on mouse melanoma and mouse mononuclear cells both *in vitro* and *in vivo*.

Methods: One hundred and ten C57BL/6 female mice were divided into 5 groups and received intraperitoneal (IP) injections of B16F10 melanoma cells followed by treatment with Bovine Cartilage using different routes of administration ( IP, oral, and IP and oral). Following a treatment period of 16 days, serum levels of Vascular Endothelial Growth Factor (VEGF) were determined by ELISA at 2, 4, and 6 hours after the last treatment dose was given. Additionally, 10 mice from each group were monitored for survival for 20 days post-treatment. Moreover, B16F10 melanoma cells and mouse mononuclear cells were incubated separately with increasing bovine cartilage concentrations for 24 and 48 hours respectively. Per cent viability was determined using the trypan blue exclusion method.

Results: A significant decrease in the serum levels of VEGF was observed in the groups treated with bovine cartilage. Moreover 20% survival rate was noted in the group treated with bovine cartilage using both oral and IP administration routes simultaneously, whereas 10% survival was noted in the groups given cartilage either by the IP or oral route. *In vitro*, total eradication of melanoma cells was observed 24 and 48 hours post-treatment with 5000µg/ml and 1000µg/ml of bovine cartilage respectively. Bovine cartilage was not toxic to mouse mononuclear cells.

Conclusion: It appears that bovine cartilage possesses anti-tumor activity. This activity seems to give better results when both routes of administration are utilized. Moreover, the anti-proliferative effect of bovine cartilage seems to be selective against tumor cells only.

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# Chapter I

## INTRODUCTION

It has been reported that bovine cartilage has several useful medical properties including the acceleration of wound healing, anti-inflammatory activity, and anti-tumor activity.

The mechanism of action underlying the anti-tumor activity of bovine cartilage is not well defined. At least four possible mechanisms have been proposed; bovine cartilage may directly induce cancer cell death, it could stimulate the immune system, it may inhibit the collagenase activity and/or may inhibit angiogenesis.

The aim of this study was to investigate some of the proposed anti-tumor mechanisms of action of bovine cartilage on mouse melanoma, namely; 1- An *in vitro* study will be performed to determine if bovine cartilage has a direct toxic effect on melanoma cells, and if so, to determine if toxicity is selective. 2- Investigate the role of angiogenesis in promoting tumor growth by determining the serum levels of VEGF in melanoma-bearing mice treated with bovine cartilage and comparing levels with that in untreated melanoma-bearing mice and 3- Comparing the duration of survival of melanoma-bearing mice treated with bovine cartilage and untreated melanoma-bearing mice.

It is anticipated that the results obtained would provide some insights on the mechanism(s) by which bovine cartilage exerts its anti-tumor effect.

## **Chapter II**

### **LITERATURE REVIEW**

#### **A- Bovine Cartilage**

The mechanism involved in normal healing of wounds was of interest for many surgeons in order to understand the biochemical time sequence of wound healing and why patients treated with cortisone exhibited fibroplasia inhibition. In this context, Lattes et.al. demonstrated that the local application of cartilage has the ability to reverse cortisone induced inhibition of wound healing (1).

##### *1- Discovery*

Influenced by both, the obscurity surrounding the surgeons and the remarkable local effect of cartilage, John Prudden, at Columbia University, NY considered testing the biological effectiveness of cartilage not only against cortisone's action but also, in healing wounds that are found in normal postoperative metabolic and endocrine conditions (1).

For this purpose, Prudden applied acid-pepsin digested bovine tracheal cartilage powder locally along with other substances known to stimulate fibroplasia such as Talcum powder, bone flour, gelatin and methionine on experimental wounds in rats. Upon obtaining 20% increase in tensile strength of closed wounds treated with bovine cartilage, Prudden described cartilage's effect as unique because none of the other tested substances exhibited increase under normal nutritional

and endocrine circumstances. Moreover, substances known to be important constituents of cartilage like chondroitin sulfates A and B did not stop the cortisone-induced inhibition of fibroplasia, whereas whole cartilage did (1).

The present experiment was repeated by Takayuki, who along with the above mentioned substances, tried the use of increased concentrations of physiological materials that have a role in accelerating tissue granulation such as fibrinogen, reconstituted collagen, and collagen derivatives including chondromucoprotein and chondroitin sulfate. None of these substances demonstrated an effect similar to that of the cartilage effect (2).

This study, along with Prudden's results, served as strong evidence for the specificity of the "cartilage effect" and further emphasized that the repair stimulating factor is present in an untreated cartilage (1, 2).

Taken into consideration the promising characteristics of locally applied cartilage, the next step was to assess, if any similar wound accelerating effect would be produced by cartilage placed at a distance from the wound. As hypothesized, subcutaneous implantation of bovine tracheal cartilage resulted in significant acceleration of incised wounds (3).

This experiment suggested that the repair stimulating factor found in cartilage is water soluble and is effective when administered by injection. Indeed, when saline extracted bovine cartilage was injected in rats at a distant anatomical site from the wound, positive results were obtained, which clarified the notion that the stimulating factor can be transported through blood. Acid mucopolysaccharide was suggested to be responsible for this biological activity (3, 4).

Additionally, parenteral administration of saline extracts of bovine cartilage resulted in similar positive outcomes when tested in different species such as dogs, rats, mice and guinea pigs. This

led Prudden to conclude that the effect of repair stimulating factor found in the cartilage is not species specific. Moreover, Prudden demonstrated that by altering the physical state of cartilage or by using young animals or different species (shark) as the main source of cartilage, the activity can be increased significantly (5).

Final clinical evaluation of cartilage's activity was to determine whether all the above obtained data would also hold true for humans. For this purpose, Prudden applied topical cartilage to more than 60 chronically non-healing and non-granulating open wounds which resulted in initiation and transformation of these wounds into granulating ones. Additionally, in 15 human volunteers 2 similar skin incisions were made on comparable structural sites, where one of the wounds was treated with cartilage whereas the other remained untreated to serve as a control. Twelve out of fifteen pairs showed 42% increase in tensile strength in the treated wounds compared to the untreated wounds. This established the ability of bovine cartilage to accelerate wound healing in humans (6, 7).

Finally, isolation of the specific chemical agent responsible for this outstanding biological activity was essential. In 1970, through vigorous studies Prudden et al. (8) arrived at a conclusion that the active component involved in wound healing is a polymer of N-acetyl glucosamine.

Later, the anti-inflammatory effect of Bovine cartilage was investigated (9). Inflammation which is an unavoidable consequence of tissue injury is the second phase of the four tightly integrated and overlapping wound healing process. Sometimes wounds do not advance to the fourth stage, which is the scar formation, but continue the inflammatory process resulting in chronic wound formation which is a major medical problem. Interestingly, in contrast to adult humans, early

fetal wound healing relatively lacks inflammation and hence heals rapidly without scar formation (10, 11).

In an attempt to reproduce the accelerating wound healing effects of bovine cartilage obtained by Prudden et. al. (6, 7), Houck and Vickers treated two groups of rats with intradermal croton oil (which is known to induce local inflammation and necrosis in rat skin) and treated one of these groups with powdered cartilage (12). Apart from decreasing the time needed for normal healing of wounds from 28 days to 15 days, topically applied cartilage significantly reduced the dermal changes that accompany inflammation including: total loss of stainable acid mucopolysaccharides from the wound, presence of high levels of endogenously synthesized glycoprotein in the ground substances of the necrotic tissues, and finally marked decrease in the amounts of insoluble collagen from the site of local injury (12). According to Houck and Vickers the inhibition of these chemical changes might be due to cartilage induced increased interaction between the wound and the circulation (12).

The anti-inflammatory capacity of Bovine Cartilage was further emphasized when a patient with psoriasis sought Prudden's help because of a non-healing ulcer that was widely spread in scaly patches on the patient's leg. Powdered cartilage was applied to the ulcer for 3 days; as a result both the ulcer and the psoriasis lesions improved. Moreover, Prudden encountered two cases of pruritus ani which were reported to be unresponsive to all topical therapies, yet responded remarkably and rapidly to cartilage powder (9).

These two events led Prudden to start systematic analysis of cartilage's anti-inflammatory activity. Throughout his investigation, different types of severe acute and chronic inflammatory and allergic cases (osteoarthritis, poison oak and poison ivy, acne, mandibular alveolitis,



hemorrhoids and fissure-in-ano, psoriasis, rheumatoid arthritis, ulcerative colitis, Regional enteritis, and progressive systemic sclerosis) were treated to evaluate cartilage's efficacy. In some cases the patients responded completely to the treatment ( Pruritus ani, poison oak and poison ivy , acne, mandibular alveolitis, and hemorrhoids and fissure-in-ano), while in others partial or no positive results were obtained (9). It can be noted that conventional methods of treating the diseases included in the study took a much longer time for a cure or partial cure to occur.

As a result, Prudden concluded that any material that has this outstanding effect on inflammation, must possess potent immunosuppressive effect as well, which was verified by the ability of cartilage to treat autoimmune and inflammatory diseases (9).

However, the mechanism by which cartilage inhibits inflammation or allergic conditions and how it is similar or different from other immunosuppressive agents is not yet known. The isolated N-acetyl glucosamine responsible for accelerating wound healing does not possess the anti-inflammatory properties of the cartilage (9). In this context, Prudden assumed that because cartilage is composed of glycoproteins and polysaccharides, it is able to cover the cellular membranes thus avoiding autoimmunity through blocking antigen-antibody reactions, without affecting resistance to infectious agents (9).

This proved to be true, when gelatin discs containing known number of *Escherichia coli* were inserted into the peritoneal cavity of two different groups of mice and the LD50 was obtained. The group treated with cartilage prior to insertion and after 4 days of insertion produced the same LD50 as the untreated group. Accordingly, Prudden concluded that bovine cartilage differs from

other immunosuppressive agents by its unique ability to manage inhibition of acute and chronic allergies without developing resistance to the infection (9).

The positive results obtained with cartilage's anti-inflammatory activity, specifically its efficacy in treating psoriasis, its record of complete non-toxicity, and its ability to treat a hopeless patient's breast cancer which had ulcerated and covered her entire chest wall, drove Prudden to test cartilage's effect in the clinical treatment of cancer (13).

For this purpose, 31 terminally ill patients each with a different type of malignancy, with whom all conventional methods of treatment had failed, were subjected to bovine cartilage treatment. Throughout the experiment, Prudden registered some significant decrease in a wide variety of intractable malignancies including cancer of lung, thyroid, pancreas, cervix, rectum, prostate, ovary, glioblastoma multiforme, and inoperable squamous cancer of the nose (13).

One case with cervical carcinoma, one with pancreatic carcinoma, and one with squamous cell cancer of the nose, resulted in a complete response to the cartilage with no recurrence even after 7 1/2, 8 and 5 years respectively; however, Prudden classified these cases as probable cure because the patients were continuing the treatment (13).

One striking case that represented complete cure was a patient with glioblastoma multiforme, who even after discontinuing the treatment, against Prudden's advice, remained clear of the disease for 5 years (13). Furthermore, several cases although presented significant early and long term positive effects, eventually died because of diseases other than cancer (13). Summing up, 11 of 31 patients had a complete positive response, 18 patients had a positive response but experienced a relapse and 2 patients had a progression of the disease. Nevertheless, it was

proposed that, in order for cartilage to be effective, treatment should start as soon as conventional therapies fail (13).

Finally, Prudden concluded that when patients experience pancreatic cancer, squamous or adenocarcinoma of the lung, glioblastoma multiforme or other malignancies where conventional treatments are known to be helpless, cartilage therapy should be considered as the primary agent in treating these diseases. He went further to add that, opposed to chemotherapy, Bovine Cartilages doesn't suppress hematological nor immunological factors (13).

## ***2- Routes of Administration and Treatment Strategy***

According to Prudden, to have maximum efficacy both oral and subcutaneous routes should be utilized, while the maintenance dosage is given orally. Treatment should start with administering cartilage by injection. This is considered to be the "loading phase". The loading phase is considered to be complete when a total of 2000 ml of a cartilage extract is injected into the patient, after which treatment is pursued by administering cartilage by the oral route (13).

Normally, patients should consume 8 capsules every 8 hours each containing 375mg of powdered cartilage. In some patients there are cancer-induced digestive problems, so in order not to disrupt the patient's appetite and hence the nutrition, deviations in the dosing timetable can take place as the patient prefers that can range from 4.5g every 12 hours to one or two capsules whenever the patient feels able to take those (13).

### ***3- Suggested Mechanism of Action***

The mechanism of action underlying the anti-tumor activity of bovine cartilage is not well defined; however, four possible mechanisms have been proposed: bovine cartilage may directly induce cancer cell death, it could stimulate the immune system, or it may inhibit collagenase activity and/or angiogenesis.

#### ***a- Acts directly on tumor cells***

Throughout Prudden's study, two patients with prostate cancer were encountered, where tumors had disappeared while they were still on oral cartilage therapy; nevertheless, recurrence of the tumor took place 6 years later. It was concluded that although cartilage might not immediately eradicate tumor cells, it possesses potent ability to arrest the growth of different types of tumors (13).

Furthermore, the indication of cartilage's ability to create steady state without direct eradication of the transformed cells' ability to form new cancer cells came from a 63 years old patient with ovarian carcinoma that was metastasized to the peritoneal and supraclavicular nodes, along with large mass in the right pelvis and massive ascites, in addition to partial upper rectal obstruction. Upon oral cartilage therapy, the mass in the right pelvis were not noticeable, and the abdominal swelling along with the ascites disappeared. Pelvic examination was normal and supraclavicular nodes disappeared as well. No intraperitoneal metastasis was evident in the diagnosis and biopsies from omentum, liver and gall bladder showed no tumor. However, upon cessation of cartilage therapy, reappearance of supraclavicular nodes took place with recurrence of ascites and pelvic mass which subsequently lead to her death (13).

Finally, proof of cartilage's direct anti-tumor activity came from *in vitro* experiments. Brian et al (14) demonstrated the anti-proliferative activity of bovine cartilage against MCF-7 human breast cancer cell line, WIDR colon cancer cell line and human myeloma cell lines 8226 and U266. Moreover, cartilage was able to inhibit the growth of several different types of human tumor biopsies taken from patients with ovarian cancer, lung cancer, breast cancer, brain cancer cervical cancer, pancreatic cancer, sarcoma, colon cancer, testicle cancer and melanoma (14).

*b-* Stimulation of the immune system

Catrix is an acidic mucopolysaccharide complex derived from bovine tracheal cartilage and contains in addition to denatured collagen, glycosaminoglycans which includes chondroitin sulfate (15). Since several studies have shown that chondroitin sulfate A and C have the capacity to augment antibody production in mice and trigger B cell proliferation *in vitro*, Rosen et. al. (15) questioned whether cartilage shares these stimulatory properties with chondroitin sulfate.

Regardless of the route of administration, cartilage enhanced the antibody response to T cell dependent and T cell independent antigens. This led Rosen et al (15) to hypothesize that, the augmenting effect of cartilage is mediated through direct stimulation of B cells or through indirect influence mediated by macrophages. Further support that the obtained result is independent of T cells, came from athymic mice which demonstrated significant enhancement in antibody production to T cell independent antigens (15). In this context, Prudden reported that bovine cartilage caused an increase in serum levels of IgM, IgG and IgA (16).

Moreover, Prudden assessed the effect of cartilage in the lymphoproliferative responses of 9 cancer patients to different mitogens (13).

It is generally accepted that the lymphoproliferative response to mitogens in patients with cancer is less than that of the normal population, and this response is suppressed even further in patients undergoing chemotherapy. Yet, lymphocytes from nine patients undergoing cartilage treatment demonstrated major elevation in their stimulation indices in response to the effects of mitogens (phytohemagglutinin, concanavalin A, and pokeweed mitogen) (13).

Additionally, Morell et al. (16) stated that Johnson reported that bovine cartilage has a potent ability to activate white blood cells and specifically macrophages.

Macrophages secrete different types of cytokines in response to invading pathogens be it microbes or transformed cells. Among these cytokines is Interleukin-12 (IL-12) which possesses strong anti-tumor and anti-metastatic properties. (17, 18) IL-12 receptors are expressed on Natural Killer cells, activated T helper cells (CD4+) and activated cytotoxic T cells (CD8+). Several studies reported the ability of IL-12 to augment the lytic activity of tumor infiltrating lymphocytes towards autologous tumor cells obtained from patients with ovarian cancer and melanoma (17, 18). Moreover, the anti-angiogenic ability of IL-12 is believed to be mediated indirectly through interferon gamma which is a potent neovascularization inhibitor (19, 20).

Two additional properties of cartilage are its ability to activate natural killer cells (NK) which provide the primary line of defense against transformed cells, and secondly the affect exerted by bovine cartilage on cells of the immune system to trigger the release of colony stimulating factors, which are known to simulate granulocyte and monocyte growth, in addition to macrophage activation (16).

c- Inhibition of collagenase activity

Tumor invasion is the dynamic relocation of tumor cells out of their original primary tissue into neighboring tissues of different types. During this process, penetration of the extracellular matrix should take place which is composed of dense meshwork of collagen, elastin, proteoglycans and glycoproteins, which act as a mechanical barrier, inhibiting cell movement. However, during special conditions like wound healing, normal tissue remodeling, inflammation and cancer, the matrix becomes permeable to cell movement (21).

Birbeck and Wheatley (22) were the first to report that interstitial collagen breakdown takes place during tumor invasion. Several experiments conducted in both animals and humans reported that collagenase activity is higher in metastatic tumors compared to their benign counterparts. Moreover, the most convincing evidence that collagenase secretion has an important role in tumor invasion comes from *in vitro* experiments where collagenase inhibitors are actually able to inhibit the tumor invasion of the extracellular matrix and hence are unable to metastasize (22, 23).

Osteosarcoma is the most known malignant tumor of bones, which are mainly made up of organic component known as collagen; on the other hand, cartilage which is found adjacent to many bones is also mainly composed of collagen, yet it is rarely penetrated by osteosarcomas. This phenomenon is also repeated with breast cancer that metastasizes to the bones. The resistance found in cartilage was attributed to the collagenase inhibitor, which is thought to be an integral part of the cartilage matrix (23).

For this purpose Kuettner et al (23), conducted an experiment in which they demonstrated that although human mammary carcinoma and osteosarcoma produce collagenase in tissue cultures, this activity is prevented by low molecular weight cationic protein derived from bovine hyaline cartilage. In this context several other experiments also reported the inhibitory activity mediated by cartilage (23).

Partial Purification of collagenase inhibitor derived from bovine cartilage was obtained by Murray et al (24) that displayed more than 60% resemblance over the first 23 residues with the inhibitor obtained from human skin fibroblasts. The sequence of collagenase inhibitor was shown to be as follow: CYS-SER-CYS-SER-PRO-VAL-HIS-PRO-GLUN-GLN-ALA-PHE-CYS-ASN-ALA-CYS-ILE-VAL-ILE-ARG-ALA-LYS-ALA-VAL-ASN-LYS-LYS-GLU-VAL-GLU- X -GLY- ASN- X -ILE-TYR-GLY-ASN-PRO-ILE-X -SER-ILE-GLN-TYR (24).

#### *d- Inhibition of neovascularization*

Apart from demonstrating its ability to inhibit collagenase activity, cartilage also plays an important role in inhibiting neovascularization.

Folkman et al (25), reported that tumor induced capillary proliferation was inhibited by new born scapular cartilage. By using rabbit cornea as an assay, implanted cartilage was able to reduce the rate of capillary proliferation produced by tumors by 75%; moreover, 28% of the tumors failed to vascularize completely. Folkman et al (25) suggested that the inhibitor secreted from the cartilage doesn't antagonize tumor angiogenic factor (TAF) but directly prevents capillary proliferation.



In 1983 Sanger et. al. (26) partially isolated TAF and in 1989 full purification was obtained by Ferrara et. al. (27) who named it Vascular Endothelial Growth Factor (VEGF). VEGF plays an important role in physiological angiogenesis, which is required for wound healing, bone formation, hematopoiesis and development. However, VEGF also has an important role in pathological angiogenesis. In order for tumors to grow and metastasize, vascular network is a must to meet their oxygen and nutrient needs and allow spreading throughout the body (26).

VEGF is thought to promote tumor malignancies through several mechanisms including vascular permeability, endothelial cell activation, survival, proliferation, migration and invasion, in addition to recruitment of progenital endothelial cells. Apart from its proangiogenic effects, VEGF has vascular independent functions as well, including its ability to suppress the functional maturation of immature dendritic cells without affecting the mature ones, and to promote tumor survival, migration and invasion through direct autocrine effect (28, 29).

Additionally, shark cartilage referred to as Neovastat (AE-941) was shown to exhibit anti-angiogenic properties when tested in the chorioallantoid membrane of chicken embryo.

Moreover, oral administration of shark cartilage blocked bFGF-induced angiogenesis in mouse models, and in the Lewis lung carcinoma model a decline of lung metastasis was observed (30).

## **Chapter III**

### **MATERIALS AND METHODS**

#### **A- Bovine Cartilage**

Bovine Cartilage used in this study was “NOW bovine Cartilage” (395 S. Glen Elyn Rd, Bloomington, IL), a commercially available bovine cartilage extract. The powdered cartilage was weighted and dissolved in sterile water. The prepared soluble cartilage was stored at -20° C.

#### **B- B16F10 melanoma cells use *in vivo***

##### ***1. Culture and preparation of injections***

The tumor cells used were the B16F10 metastatic melanoma cells, which are syngeneic with the C57BL/6 mice that were used in this study. Melanoma cells were maintained as monolayers *in vitro* in culture medium (RPMI-1640 supplemented with 1% L-Glutamine, 1% Penicillin-Streptomycin and 10% Fetal Bovine Serum) and incubated at 37° C in a 5% CO<sub>2</sub> incubator (Thermo scientific, Forma, series II water jacket, and CO<sub>2</sub> incubator). When confluent growth was achieved, the medium was discarded and the cells were washed twice with PBS and detached with 1x trypsin (2.5% Trypsin 10x in HBSS without Calcium or Magnesium). Fresh medium was then added to the cells and the suspension was centrifuged at 900 rpm for 5 minutes (Refrigerated centrifuge, Biofugestratos, Heraeus). The supernatant was discarded and the pellet

was re-suspended in fresh medium. The suspension was dispensed into T75 plates for culture again.

On the day of tumor induction into mice, the melanoma cells were washed with PBS, detached with 1x trypsin, and re-suspended in fresh media. The viable cell count was determined by counting viable and dead cells using the trypan blue exclusion and 'Neubauer chamber' method. The cell suspension was then centrifuged at 900 rpm for 5 minutes, the supernatant was discarded and the pellet was suspended in 25 ml of RPMI-1640 medium.

## ***2. Challenge of mice with tumor cells and treatment***

One hundred and ten C57BL/6 female mice, four to six weeks old, were obtained from the Animal Care Facility at the American University of Beirut after obtaining the approval of Institutional animal care and use committee (IACUC).

All mice were then injected intraperitoneally (IP) with 3.4 million cells/Kg suspended in 0.5 ml medium and were divided into five groups as shown in **Table 1**.

Group I served as a control and received only one injection of B16F10 melanoma cells. Group II also served as a control and received two days later post B16F10 melanoma cells challenge, daily IP sterile water injection. Group III received daily IP injection of Bovine cartilage at a dose of 75 mg/ml in 0.4 ml solution two days post tumor challenge. Group IV received daily Bovine cartilage treatment administered through drinking water at a dose of 37mg/mouse, two days later post tumor injection, and the drinking water was changed every three days. Group V received one injection of B16F10 melanoma cells and two days later Bovine cartilage was administered daily through IP injection at a dose of 37.5mg/ml in 0.4ml and through drinking water at dose of

18.5mg/mouse, the drinking water was changed every three days. All groups received treatment for 16 consecutive days.

In order to evaluate the efficacy of the different therapeutic strategies used, ten mice from each group were monitored for twenty days, time of death was noted and survival percentage was assessed. Dead mice were dissected to confirm that death was due to the tumor.

### **C. Procurement of Specimens**

At 2, 4 and 6 hours post-injection (after the last dose of Bovine Cartilage on day 16), 4 mice from each group were anesthetized each with a 0.5 ml of a mixture of 0.12 ml ketamine (12 mg/ml), 0.03 ml xylazine (1.2 mg/ml) and 0.35 ml sterile saline. The mice were then dissected and blood was collected by cardiac puncture. Blood from each group was pooled and serum was separated and stored at -4° C.

### **D. Vascular Endothelial Growth Factor (VEGF) Quantification by the Enzyme-Linked Immunosorbant Assay (ELISA)**

The kit included the VEGF standard, detection antibody (biotinylated anti-mouse VEGF), Horseradish peroxidase-Streptavidin (HRP-Streptavidin), assay diluent A, assay diluent B, wash buffer concentrate (20X), TMB (tetramethylbenzidine) One-Step substrate reagent and stop solution.

- Working wash buffer” was prepared by suspending 20 ml of “10x wash buffer” in 400 ml distilled water.
- 1x assay diluent B was prepared by mixing 5 ml of diluent B (5x) with 20 ml distilled water.

- Four hundred  $\mu\text{l}$  of “assay diluent A” were added to the vial containing the antigen (VEGF) resulting in a 25 ng/ml standard.
- A serial dilution of the antigen (VEGF) standard (1000, 400, 160, 64, 25.6, 10.24 and 4.1 pg/ml) was prepared in a set of eight 5 ml (12 x 75) polypropylene tubes; 960  $\mu\text{l}$  of assay diluent A were added to the first tube and 300  $\mu\text{l}$  of the same diluent to the seven remaining tubes.
- Forty  $\mu\text{l}$  of the antigen standard (25 ng/ml) were added to the first tube to yield a concentration of 1000 pg/ml.
- A series of 1:2 dilutions was conducted by transferring 200  $\mu\text{l}$  from the first tube into the second and so on up to the seventh tube. No antigen was added to the eighth tube that served as the zero standards and had a concentration of 0 pg/ml.
- Mice sera, previously collected and frozen, were thawed in a water bath at 37° C.
- Detection antibody concentrate was prepared by adding 100  $\mu\text{l}$  of 1x diluent B into the vial containing the antibody. Then the detection antibody concentrate was diluted 80-fold by adding 100  $\mu\text{l}$  of the concentrate to 7.9 ml of 1x diluent B.
- HRP-Streptavidin concentrate was diluted 160-fold by adding 75  $\mu\text{l}$  of the concentrate to 11.925 ml of 1x assay diluent B.
- One hundred  $\mu\text{l}$  of sample to be tested and 100ul of standard were each added into the appropriate wells of the plate (each sample and standard was run in duplicate).
- The plate was then incubated at room temperature for 2<sub>1/2</sub> hours. The wells were then washed four times using the 1x wash buffer to remove excess antigen.
- Hundred  $\mu\text{l}$  of detection antibody were added to each well and the plate was incubated at room temperature for 1 hour.

- The wells were again washed four times to remove residual unbound detection antibodies. 100 µl of HRPStreptavidin were added to each well, the plate was incubated at room temperature for 45 minutes and then washed four times.
- Hundred µl of the TMB One-Step substrate reagent were added to each well and the plate was incubated at room temperature for 30 minutes in the dark.
- Finally, 50 µl of the stop solution were added to each well of the plate and at this point the color changed from blue to yellow. The absorbance was read at 450 nm using the Bio-Tek /ELx800 micro-plate reader.

## **E. In vitro Evaluation of Bovine Cartilage Effect**

### ***1- B16F10 melanoma cells***

B16F10 melanoma cells were maintained as monolayers in a culture medium containing RPMI-1640, 10% FBS, 1% L-Glutamine and 1% Pen-Strep and incubated in T25 or T75 flasks at 37<sup>0</sup>C & 5% CO<sub>2</sub>. Once confluent growth was achieved, the medium was discarded, the cells were washed twice with PBS and detached with 1x trypsin (2.5% Trypsin 10x in HBSS without Calcium or Magnesium). Fresh medium was added to the cells and the suspension was centrifuged at 900 rpm for 5 minutes (Refrigerated centrifuge, Biofugestratos, Heraeus). The supernatant was discarded and the pellet was re-suspended in fresh medium. The suspended cells were counted and seeded in 24-well plates, with a seeding density of 2x10<sup>4</sup> cells in 500µl per well, and were left to adhere for 24 hours. After 24 hours, 500µl of culture medium was added to the control wells and 500µl of five different bovine cartilage concentrations (5µg/ml, 10µg/ml, 100µg/ml, 1000µg/ml and 5000µg/ml) were added to each of the remaining wells. The samples

were run in duplicates to ensure the accuracy of the results. Cells were then incubated at 37° C in a 5% CO<sub>2</sub> incubator for 24 and 48 hours respectively.

After 24 and 48hrs of incubation, the medium with bovine cartilage was removed from the wells and the cells were washed twice with 500µl PBS and detached with 0.05% trypsin. Fresh medium was added to the cells and the suspension was centrifuged at 300\*g (900 rpm) for 5 minutes. The supernatant was discarded and the pellet was re-suspended in fresh medium. The viable cell count was determined by the trypan blue exclusion method using the “Neubauer chamber.

## ***2- Mouse mononuclear cells***

Seven healthy Black female C57BL/6 mice were sacrificed and spleens were collected and pooled aseptically. Spleens were finely cut into small pieces with sterile blades in petri dishes, under a biological safety hood. With the use of a plunger, grinding of the pieces was carried out. The pieces were smashed by adding 3ml saline and 1ml RBC lysis solution and a cell suspension was prepared containing the mononuclear cells. The cell suspension was then passed through a sterile cell strainer having a 70µm pore size into a 50 ml conical tube. Later, the cell suspension was centrifuged at 1500 rpm for 15 min. The resulting supernatant was discarded and the pellet was re-suspended in 5ml of fresh media containing RPMI, 1% L-Glutamine and 10% FBS. The suspended cells were counted and seeded in 24-well plates, with a seeding density of  $3 \times 10^6$  cells in 500µl per well. Then 500 µl of five increasing bovine cartilage concentrations (5µg/ml, 10µg/ml, 100µg/ml, 1000µg/ml and 5000µg/ml) were then added to the different wells and one control well was left with no bovine cartilage (only 500 µl culture medium was added). All wells were run in duplicates to insure accurate results. Cells were then incubated for 48 hours at 37° C in a 5% CO<sub>2</sub> incubator. After incubation, the cell cultures were collected and centrifuged at 1500

rpm for 15 min. The resulting supernatant was discarded and the pellet was re- suspended in 5ml of fresh media. Viable and dead cell count was determined by the trypan blue exclusion method using the 'Neubauer chamber'.

#### **F- Statistical analysis**

Whenever applicable, data were expressed as Mean  $\pm$  SD. Mice survival was evaluated by generating Kaplan–Meier survival curves. The unpaired student T-test was implemented to assess the sample variations between groups using the Graphpad online software. Results were considered to be statistically significant when P value was  $<0.05$ .



## Chapter IV

### RESULTS

#### A. Serum levels of VEGF

##### ***1- Group III: given daily IP injections of bovine cartilage for a period of 16 days.***

The serum levels of VEGF in group III at 2 hours post-injection was 12212.11pg/ml, however the VEGF levels in the control group that received daily IP sterile water, at 2 hours post-injection were out of range, because of the high levels of VEGF (higher than the highest standard's absorbance); hence comparison could not be made at 2 hours post injection. However, at 4 and 6 hours post- injection there was a profound decrease in VEGF level, in mice treated with daily IP injection of bovine cartilage at dose of 0.4 ml of a 75mg/ml of Bovine Cartilage solution (**Figure 1**).

##### ***2- Group IV: given Bovine Cartilage orally for a period of 16 days***

The serum levels of VEGF in group IV, compared to those in untreated control, group I, decreased profoundly at 2 , 4 and 6 hours after the last bovine cartilage treatment administered orally (**Figure 1**).

##### ***3- Group V: Bovine Cartilage administrated by both IP and oral routes given for a period of 16 days.***

The serum levels of VEGF in group II (control) that received distilled water as well as in cartilage treated group V, administered IP and orally, at interval of 2 hours post-injection were out of range (higher than the highest standard's absorbance), hence comparison was not made at

interval of 2 hours. The serum levels of VEGF in the treated mice decreased slightly but remained significantly higher than that of the control at 4 hours post cartilage administration. Profound decrease was obtained in the mice treated with bovine cartilage after 6 hours post-administration (**Figure 1**).

## **B. Mice survival rates**

### ***1- Group III: given bovine cartilage IP***

The control group, given IP injections of sterile water, were all dead by day 17 (0% survival). By day 20, nine out of ten mice treated with IP injection of bovine cartilage at a dose of 0.4ml of 75mg/ml were dead (10% survival). The remaining one mouse showed complete response to cartilage treatment (probable cure) (**Figure 2**).

### ***2- Group IV: given bovine cartilage orally***

The control group, given one injection of melanoma cells only, was dead by day 18 (0% survival). By day 20, nine out of ten mice treated with bovine cartilage through drinking water at a dose of 37mg/mouse were dead (10% survival) (**Figure 2**).

### ***3- Group V: given bovine cartilage both IP and orally***

The control group, given daily IP injections of sterile water, was dead by day 17 (0% survival). By day 20, eight out of ten mice treated with bovine cartilage both IP at dose of 0.4ml of 37mg/ml and orally at dose of 18.5mg/mouse were dead (20% survival) (**Figure 2**).

The survival results were further evaluated by generating the Kaplan Meier survival curves, showing the probability of survival in a given period of time (**Figure 3**). The p-values were calculated to assess the statistical significance of the results obtained. P-values  $\leq 0.05$  were considered statistically significant.

The survival results obtained from group III, given bovine cartilage IP, and group IV given bovine cartilage orally, as compared to their controls, treated with daily IP injections of sterile water and injected with only melanoma cells respectively, showed no statistical significance (P values of 0.931 and 0.482 respectively). However, in group V given bovine cartilage both orally and IP, showed statistically significant survival results (P value of 0.047) when compared to daily IP injection of sterile water-treated control (**Figure 4**).

### **C. In Vitro results**

#### ***1- Viability of B16F10 melanoma cells after 24 hours***

Compared to the control well, there was a decrease in the melanoma cell count in the well treated with 500 $\mu$ l of 5 $\mu$ g/ml of bovine cartilage. Significant decrease was obtained in the number of viable melanoma cells treated with 500 $\mu$ l of 10  $\mu$ g/ml, 100  $\mu$ g/ml, and 1000  $\mu$ g/ml of bovine cartilage concentrations respectively. Total eradication of melanoma cells were obtained in the wells treated with 500 $\mu$ l of 5000 $\mu$ g/ml of bovine cartilage concentration (**Figure 5**).

#### ***2- Viability of B16F10 melanoma cells after 48 hours***

Compared to the control well, significant decrease in viable melanoma cell count was obtained in the wells treated with 500 $\mu$ l of 5  $\mu$ g/ml, 10  $\mu$ g/ml, and 100  $\mu$ g/ml of bovine cartilage concentrations respectively. Total eradication of melanoma cells were obtained in the wells

treated with 500µl of 1000 µg/ml and 5000 µg/ml of bovine cartilage concentrations respectively (**Figure 5**).

The *In Vitro* experiment conducted on the viability of B16F10 melanoma cells was repeated three times and consistent results were obtained.

### ***3- Viability of mouse mononuclear cells after 48 hours***

Compared to the control well, the viability of mouse mononuclear cells remained relatively stable throughout the first four bovine cartilage concentrations (5 µg/ml, 10 µg/ml, 100 µg/ml, and 1000 µg/ml); however a significant decrease was seen with the highest concentration which is the 5000 µg/ml (88% survival) (**Figure 6**).

### **D. Microscopic examination of specimens taken from dead mice**

Before dissection, Palpable solid tumor growth at site of injection was observed along with enlarged organs (**Figure 7a**). Upon dissection of dead mice, tumor growth with necrotic organs was observed (**Figure 7b**). A small piece of a tumor mass was taken and placed on a slide with a drop of trypan blue. The cells were observed under the microscope (100x) and large cancer cells were detected which further confirmed that the death of mice were due to disseminated cancer cells.

However one mouse from group III which might have shown a complete response to cartilage did not show any solid tumor growth externally (**Figure 7c**). Moreover, upon dissection no necrotic organs were visible (**Figure 7d**). Blood from the dissected mouse was placed on a slide with a drop of trypan blue and a blood smear was performed. The smear was observed under the

microscope (100x). No Large cells similar to those seen in the previous cases were observed, which further led to the conclusion that this mouse might have undergone probable cure.

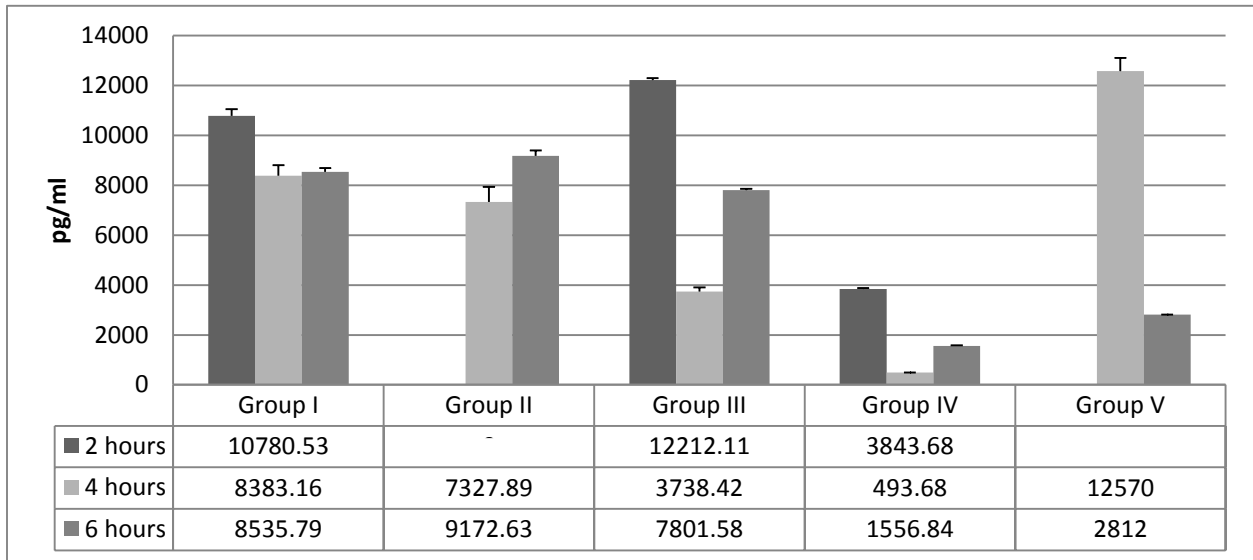
**Table 1:** The protocol followed in treating different groups of mice.

Groups Days	Group 1	Group 2*	Group 3*	Group 4**	Group 5***
Day 0	Challenge with B16F10 melanoma cells 3.4 million cells/kg in 0.5ml				
Day 3	-	Sterile water	Bovine Cartilage	Bovine cartilage	Bovine cartilage
Day 4	-	Sterile water	Bovine Cartilage	-	-
Day 5	-	Sterile water	Bovine Cartilage	-	-
Day 6	-	Sterile water	Bovine Cartilage	Change drinking water	Change drinking water
Day 7	-	Sterile water	Bovine Cartilage	-	-
Day 8	-	Sterile water	Bovine Cartilage	-	-
Day 9	-	Sterile water	Bovine Cartilage	Change drinking water	Change drinking water
Day 10	-	Sterile water	Bovine Cartilage	-	-
Day 11	-	Sterile water	Bovine Cartilage	-	-
Day 12	-	Sterile water	Bovine Cartilage	Change drinking water	Change drinking water
Day 13	-	Sterile water	Bovine Cartilage	-	-
Day 14	-	Sterile water	Bovine Cartilage	-	-
Day 15	-	Sterile water	Bovine Cartilage	Change drinking water	Change drinking water
Day 16	-	Sterile water	Bovine Cartilage	-	-
Day 17	-	Sterile water	Bovine Cartilage	-	-
Day 18	-	Sterile water	Bovine Cartilage	Change drinking water	Change drinking water

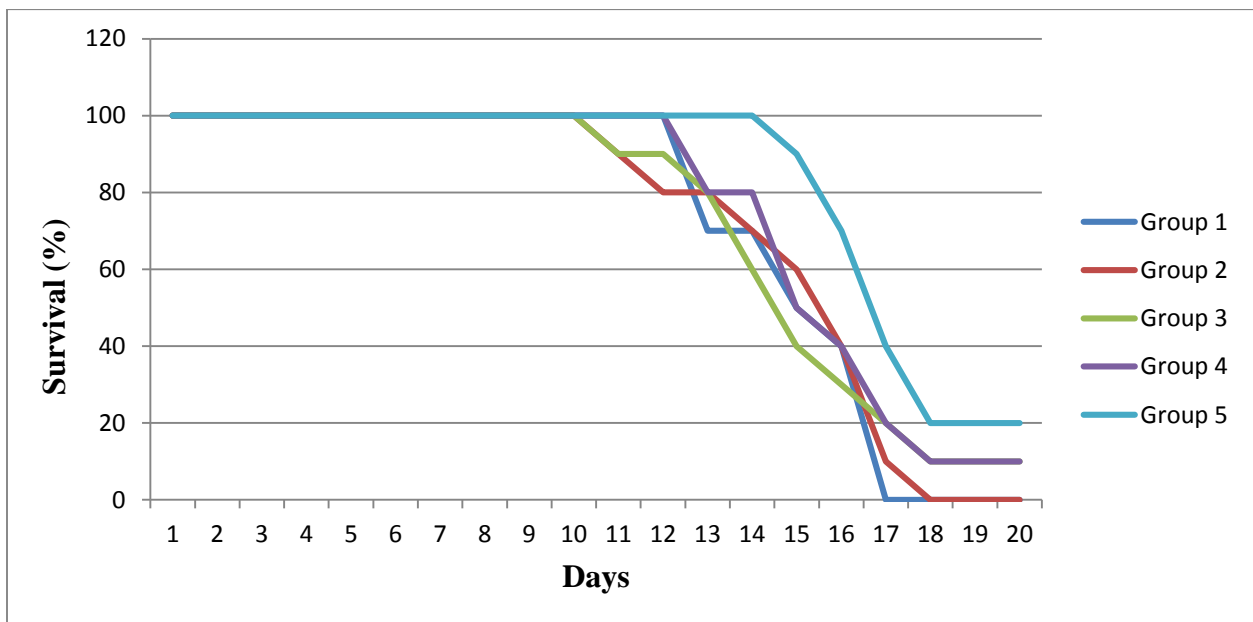
\*: Intraperitoneal injection (IP); Sterile water (0.4mL/mouse), Bovine cartilage (0.4ml of 75mg/ml)

\*\* : Bovine cartilage through drinking water (37mg/mouse), changed at 3 days intervals

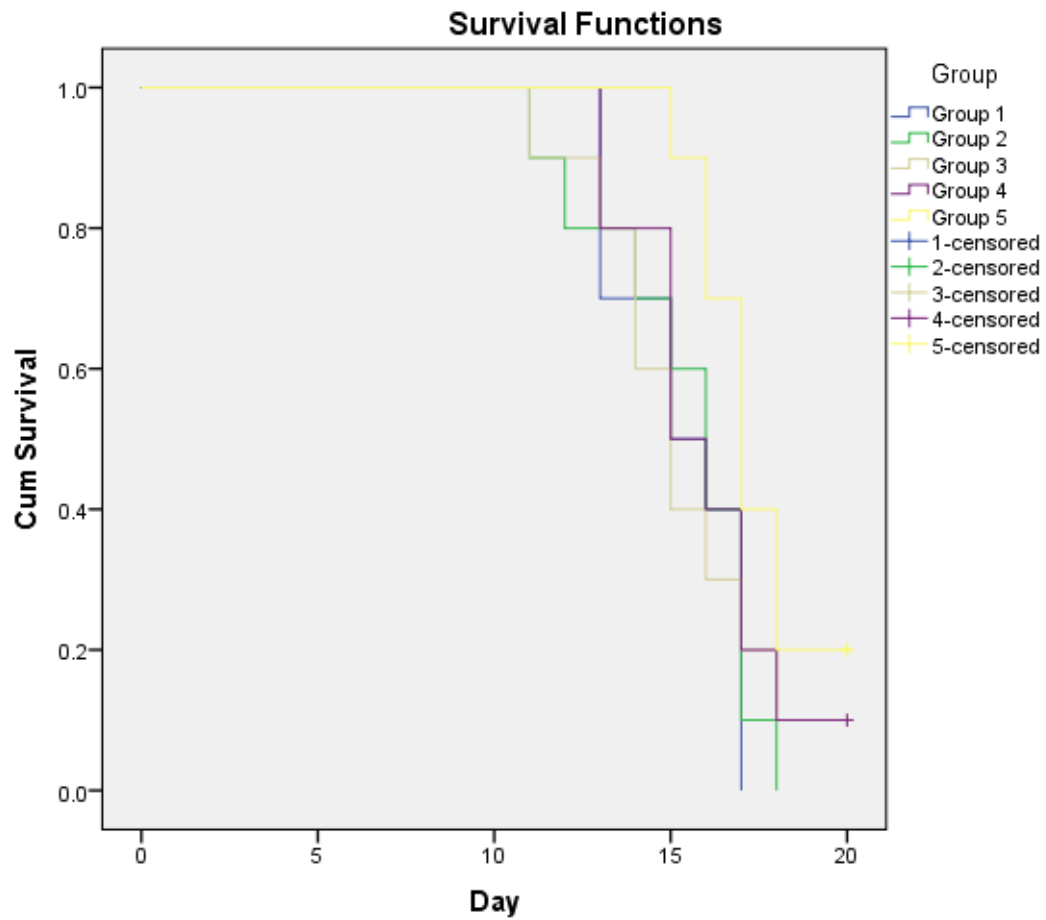
\*\*\*: Bovine cartilage IP ( 0.4 ml of 37.5mg/ml) and through drinking water (18.5mg/mouse)



**Figure 1:** Serum VEGF levels of the 5 different groups of mice after 16 days of bovine cartilage treatment at 2, 4 and 6 hours after the last treatment as detected by ELISA. In group II and V, VEGF levels at 2 hours after the last bovine cartilage administrative dose were out of range.

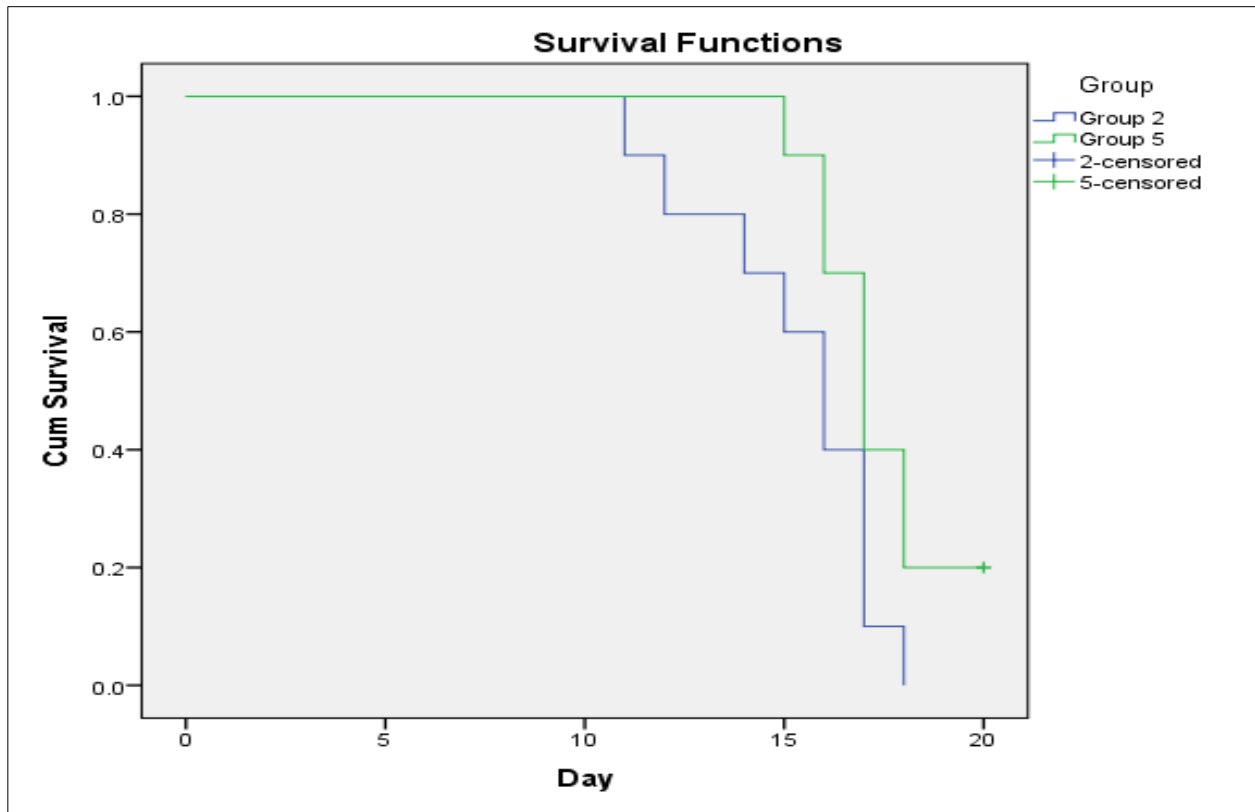


**Figure 2:** Survival rate of the 5 different groups of mice followed over a period of 20 days. By day 19, 9 out of 10 mice in group III and IV and 8 out of 10 mice in group V were dead (10% and 20% survival rates respectively), yet none of the mice in the control groups (I and II) survived beyond day 19 (0% survival).

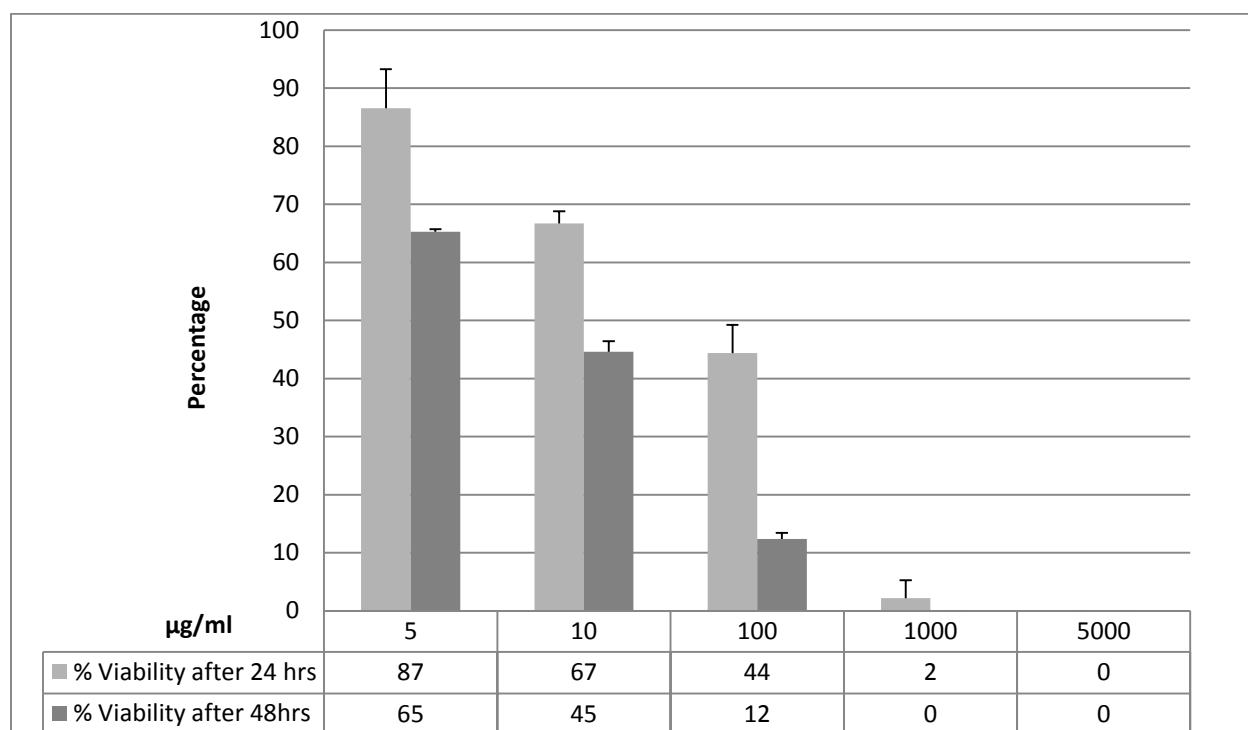


**Figure 3:** Kaplan Meier survival curve, for the five groups. When group III and IV were compared to their controls, group II and group I respectively, did not show any statistically significant survival rates, P values were 0.931 and 0.482 respectively. Significance was considered when P value <0.05

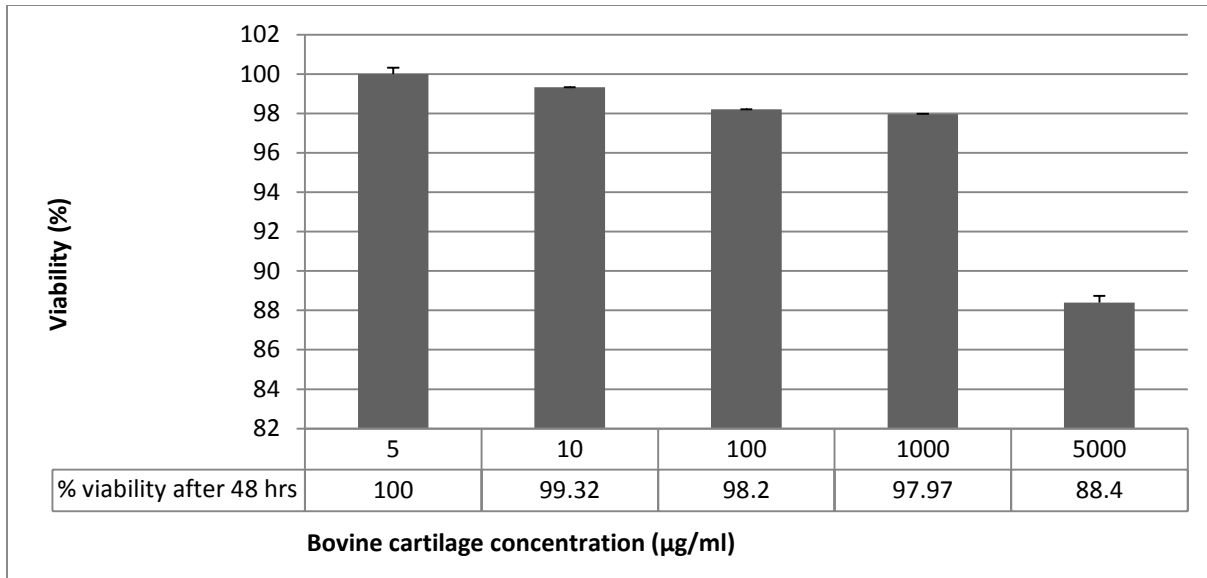




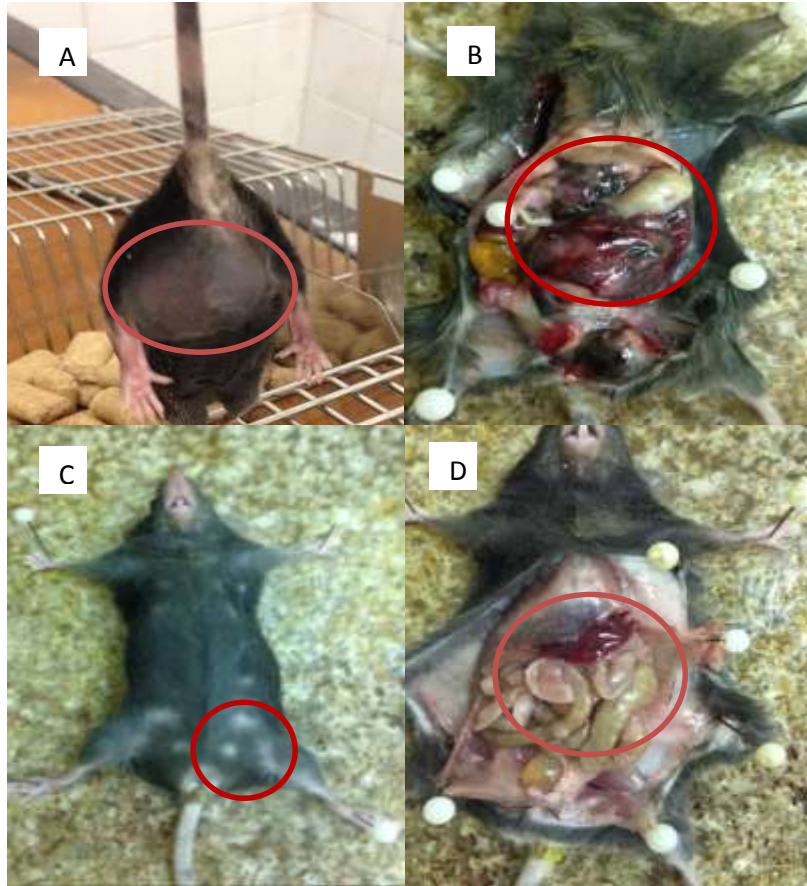
**Figure 4:** Kaplan Meier survival curve, for the groups II and V. When V was compared to its control, group II, statistically significant survival rate was obtained, P value was 0.047. Significance was considered when P value <0.05



**Figure 5:** Assessing the effect of Bovine cartilage on the growth B16F10 melanoma cells *In Vitro* after 24 and 48 hours. Melanoma cells growth was inhibited completely at 1000µg/ml at 48 hours and at 5000 µg/ml after 24hrs. Regression of growth was observed with lower concentrations as well.



**Figure 6:** Assessing the effect of Bovine cartilage on the growth of mouse mononuclear cells *In Vitro* after 48 hours. The number of viable mouse mononuclear cells remained relatively stable with increasing concentrations of bovine cartilage and only a significant decrease was seen at 5000µg/ml.



**Figure 7:** Showing presence or absence of tumor in mice (A) Palpable solid tumor growth at site of injection, (B) Necrotic organs, (C) Absence of Palpable solid tumor growth at site of injection, (D) Absence of necrotic organs and solid tumor growth at site of injection.

## Chapter V

### DISCUSSION

The anti-inflammatory effect of bovine cartilage and its ability to cure a patient's ulcerated breast cancer drove Prudden to assess cartilage's effect in the clinical treatment of cancer (9, 13).

The effectiveness of bovine cartilage in treating cancer was reported in Prudden's publication "The Treatment of Human Cancer with Agents Prepared from Bovine Cartilage" which documented an 11-year study of 31 terminally ill patients. Throughout the study, Prudden registered some significant decrease in a wide variety of intractable malignancies, and all the patients except one survived longer than the time predicated by their original doctors (13).

Although these results were promising, yet most scientists chose not to investigate further the anti-tumor activity possessed by bovine cartilage. Few *in vitro* and *in vivo* studies were conducted, but none of the *in vivo* published studies assessed the effect of bovine cartilage in cancer models (13-15, 24-26, 30, 32).

For this purpose this study was carried out, in an attempt to determine the effect of bovine cartilage on tumor growth *in vivo*, on serum level of the pro-angiogenic cytokine VEGF in tumor-bearing mice, and on the survival of mouse melanoma and mononuclear cells *in vitro*.

Among the different proposed mechanisms of action underlying the anti-tumor activity of bovine cartilage is its ability to act directly on tumor cells. This anti-tumor activity of bovine cartilage was evaluated *in vitro* when B16F10 melanoma cells were subjected to bovine cartilage

treatment. Our results indicated that there was a decrease in the viability of melanoma cells treated with bovine cartilage and this decrease was enhanced with time of exposure and amount of bovine cartilage added, reaching a total eradication of tumor cells after 48 hours of exposure with 1000ug/ml of bovine cartilage . These obtained results were consistent with those obtained by Brian et al. (14) who demonstrated the anti-proliferative activity of bovine cartilage against several tumor cell lines using the same bovine cartilage concentrations except for 5µg/ml. Moreover, when bovine cartilage was tested on mouse mononuclear cells, only significant decrease in the viability of mouse mononuclear cells was seen with 5000µg/ml after 48 hours of incubation; in contrast to melanoma cells, where total eradication of tumor cells was obtained at 1000µg/ml after 48 hours. This obtained result might further emphasize the function of bovine cartilage as a strong biological modifier, because although it is toxic to cells, but this toxicity is selective only to tumor cells. Moreover, we tested the selective toxicity of bovine cartilage *in vivo*, where mice received bovine cartilage injections for ten days and did not show any weight, skin or hair loss, for 3 months of observation, which were our markers for toxicity. Finally, our results are also consistent with Prudden's result where he reported that bovine cartilage presents no record of toxicity in humans and animals.

Next, the anti-angiogenic activity of bovine cartilage which is again one of the proposed mechanisms of action underlying the anti-tumor activity was evaluated *in vivo*. Two, 4, and 6 hours after the last bovine cartilage treatment, significant decrease in VEGF levels were obtained in group III and IV which received bovine cartilage IP and orally respectively; whereas in group V which received Bovine Cartilage treatment both orally and IP significant decrease was obtained only after 6 hours post treatment. These results were consistent with Folkman et al. (25) who demonstrated that an inhibitor secreted from bovine cartilage was able to directly inhibit

tumor capillary proliferation, and with Langer et al. (31) who purified a fraction from bovine cartilage with a molecular weight of 16,000, and demonstrated that it possesses the ability to inhibit cancer induced neovascularization.

Moreover, an association was made between the survival rates of each group and the obtained VEGF levels. Group III, which received Bovine Cartilage IP, showed significant decrease in VEGF levels after 2, 4, and 6 hours post-injection, yet the 10% survival rate compared to the control was not significant. Nonetheless, one mouse from this group showed complete response to bovine cartilage therapy even after discontinuing the treatment, similar to one of Prudden's patients who were completely cured (13). Additionally, the case of this mouse resembles the situation where some cancer patients respond to therapy whereas others don't. The underlying cause of this incident might be multifactorial including the composition of the normal microbial flora and the important role played by some specific microorganisms present in it. These microorganisms might inhibit tumor development through immune system activation and help promote complete response to cancer therapies (33, 34).

Next, significant decrease in VEGF levels at 2, 4 and 6 hours post treatment was seen in group IV, which received bovine cartilage orally. These results were somehow consistent with the route of administration utilized by Prudden in treating some of his patients, including the one who showed complete response to cartilage treatment (13); Nevertheless, the 10% survival rate was not significant compared with the control. This might be due to the fact that not enough cartilage was absorbed by each mouse. To give the desired result, each mouse should consume a daily 37mg of bovine cartilage (the equivalent of human dose advised by Prudden), but since the treatment for this group was dissolved in their drinking water, the amount taken by each mouse

was impossible to be controlled, and hence we weren't able to ensure that each mouse took the desired dose of bovine cartilage.

It should also be kept in mind that, although tumor vascularization is a vital processes for cancer growth and spreading, yet cancer cells have other mechanisms to multiply and metastasize, which might further explain why in spite of significantly decreased VEGF levels, the survival rates in group III and IV were not significant (35).

Finally, group V which received bovine cartilage IP and orally showed significant survival rate (20%) compared to the control group. This result was consistent with the report of Romano et al (32), who stated that the probable cause of the decreased cure rate obtained with his patients might be due to the utilization of the subcutaneous route only, while Prudden advised using both subcutaneous and oral administrative routes to maximize bovine cartilage's effect. This might also explain why mice in group III which received only IP injections of bovine cartilage did not show significant survival rates. Moreover, the reason behind the delayed but significant decrease of VEGF levels might be due to the decreased administrative dose of bovine cartilage divided between oral and IP. Also, it is important to note that apart from inhibiting neovascularization, bovine cartilage has other anti-tumor mechanisms, which might further explain why even though there was a delay in an apparent decrease in VEGF levels, the survival rate was significant.

In conclusion, from our results, it appears that bovine cartilage possesses selective anti-tumor activity. This activity seems to give better results when both routes of administration are utilized as suggested by Prudden. However, it is not clear yet whether the oral route is needed to make cartilage treatment an effective one. Moreover, improved protective and therapeutic results could



be achieved by increasing the population number and by using more accurate oral administrative methods.

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