

Original Research Paper

The Effect of Bovine Cartilage on the Growth of Mouse B16F10 Melanoma Cells *in vitro* and *in vivo*

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Article history

Received: 23-08-2016

Revised: 18-10-2016

Accepted: 20-10-2016

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Abstract: Earlier reports indicated that Bovine Cartilage (BC) had anti-tumor effects but only a few *in vitro* and *in vivo* investigations were conducted to assess its mechanism of action. The aim of this study was to investigate some of the proposed mechanisms of action of BC on mouse melanoma cells both *in vitro* and *in vivo* and to determine if its effect on tumor cells is selective. One hundred and ten mice were divided into 5 groups and received Intraperitoneal (IP) injections of melanoma cells followed by treatment with BC using different routes of administration. Following a daily treatment period of 16 days, serum levels of VEGF and IL-12 were determined by ELISA at 2, 4 and 6 h after the last treatment dose. Additionally, 10 mice from each group were monitored for survival for 20 days post-last treatment. Moreover, melanoma and mouse mononuclear cells were incubated separately with increasing BC concentrations for 48 h and percent viability was determined. A significant decrease in the serum levels of VEGF and significant increase in the serum levels of IL-12 were observed in the groups treated with BC. Moreover 20% survival rate was noted in the group treated with BC both orally and IP, whereas 10% survival was noted in the groups given BC either IP or orally. *In vitro*, total eradication of melanoma cells was observed with 1000 $\mu\text{g mL}^{-1}$ of BC. BC was not toxic to mouse mononuclear cells. The *in vivo* anti-tumor effect of BC was best observed when combined IP and oral doses were given and it appears that two of its actions are by activating macrophages as indicated by increases in IL-12 levels and blocking angiogenesis as indicated by decreases in VEGF levels. Finally BC showed selective toxicity against melanoma cells.

Keywords: B16F10 Melanoma Cells, Bovine Cartilage, VEGF, IL-12

Introduction

The use of Bovine Cartilage (BC) as a treatment for several medical conditions has been around for more than 40 years. Dr. Prudden was the first to assess the ability of BC to accelerate healing in experimental wounds (Prudden *et al.*, 1975; Takayuki, 1960; Prudden *et al.*, 1962). The obtained positive results drove Prudden to test the accelerating effect of BC on several different laboratory animals and on humans, where in both cases results were significant (Prudden *et al.*, 1964; Prudden, 1964; Prudden and Allen, 1965).

A study conducted by (Houck *et al.*, 1961) showed that BC had potent anti-inflammatory activity which was further emphasized, when a patient with psoriasis and a non-healing ulcer markedly improved upon BC

treatment. Hereafter several cases of acute and chronic inflammations and skin allergies were treated with BC (Prudden and Balassa, 1974).

The initial decision to test BC in the clinical treatment of cancer was based on its efficiency in the treatment of psoriasis, on its record of being non-toxic and on its ability to cure a patient's ulcerated breast cancer. The effectiveness of BC in treating cancer was reported for 31 terminally ill patients. There were some significant decreases in a wide variety of intractable malignancies and all the patients except one survived longer than the time predicted by their clinicians (Prudden, 1985).

The mechanism of action underlying the anti-tumor activity of BC is not well defined. At least four possible mechanisms have been proposed; BC may directly induce

cancer cell death (Prudden, 1985; Durie *et al.*, 1985), it could stimulate the immune system (Rosen *et al.*, 1998; Morell and Daniel, 2014), inhibit collagenase activity (Kuettner *et al.*, 1977; Murray *et al.*, 1986) and/or inhibit angiogenesis (Folkman *et al.*, 1971; Langer *et al.*, 1976).

The current study was carried out in an effort to investigate some of the proposed anti-tumor mechanisms of action of BC on mouse melanoma; and for this purpose an *in vitro* study was performed to determine if BC had a direct toxic effect on melanoma cells and if so, to determine if its toxicity is selective. Moreover, an *in vivo* study was done to determine survival rates of B16F10 melanoma-bearing mice and their serum levels of IL-12 and VEGF treated with BC compared to untreated controls.

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

Materials and Methods

In vivo Studies

BC used was “NOW Bovine Cartilage” (395 S. Glen Elyn Rd, Bloomington, IL), a commercially available extract. The powdered BC was weighed, dissolved in sterile distilled water and stored at -20°C until used.

B16F10 Melanoma Cells

B16F10 metastatic melanoma cells are syngeneic with the C57BL/6 mice. They are adherent cells and were maintained as monolayers *in vitro* in RPMI 1640 culture medium supplemented with 1% L-Glutamine, 1% Penicillin-Streptomycin and 10% Fetal Bovine Serum (Lonza, B-4800 Verviers, Belgium) and incubated at 37°C in a 5% CO₂ incubator.

On the day of tumor induction into mice, the melanoma cells were detached with trypsin (2.5% trypsin in 10× in HBSS without calcium or magnesium, Lonza, B-4800 Verviers, Belgium). The viable cell count was determined by trypan blue exclusion method using a Neubauer Chamber and re-suspended in RPMI-1640 medium. The amount of B16F10 melanoma cells injected intraperitoneally (IP) was 3.4 million cells/Kg suspended in 0.5 mL RPMI-1640.

Challenge of Mice with Tumor Cells and Treatment

The use of mice in this study was approved by the Institutional Animal Care and Use Committee (IACUC) at the Faculty of Medicine, American University of Beirut. One hundred and ten C57BL/6 female mice, 6 to 8 weeks old, were injected IP with melanoma cells at day 0 and divided into 5 groups (twenty-two mice in each). After 2 days post melanoma injection, BC treatment was given to the five groups as shown in Table 1. Two groups served as controls; one group remained untreated and served as a control for oral BC group. Another group

received daily IP injection of sterile water at a dose of 0.4 mL/mouse and served as a control for IP BC and IP and oral BC groups. The treatment was given for 16 consecutive days.

Procurement of Specimens

On day 16 and at 2, 4 and 6 h after the last BC treatment, four mice from each group were anesthetized then dissected and blood was collected by cardiac puncture. Blood from each group was pooled and serum was separated and used for Vascular Endothelial Growth Factor (VEGF) and Interleukin 12 (IL-12) quantification. The remaining 10 mice from each group were monitored for 20 days post-last treatment to determine the survival rate. Upon death, the mice were dissected to confirm that death was tumor related.

VEGF and IL-12 Quantification

VEGF mouse ELISA kit (Abcam, ab100751, USA) and IL-12p70 mouse ELISA kit (Abcam, ab119531, USA) were used to determine the serum levels of VEGF and IL-12, respectively. The procedures were performed according to the manufacturers' protocol.

In Vitro Evaluation of BC Effect

Culture of B16F10 Melanoma Cells in the Presence of BC

B16F10 Melanoma cells were counted and seeded in 24-well plates, (seeding density of 2×10^4 cells/500µl/well), then incubated for 24 h at 37°C and 5% CO₂. After incubation, 500 µL of five different BC concentrations (5, 10, 100, 1000 and 5000 µg mL⁻¹) were added to the different wells. No BC was added to the control wells.

The samples were run in duplicates. Wells were then incubated at 37°C in a 5% CO₂ incubator for 48 h. After incubation, melanoma cells were detached with 1× trypsin, then suspended in culture medium and a viable cell count was determined.

Culture of Mouse Mononuclear Cells (MNC) in the Presence of BC

Seven healthy female C57BL/6 mice were sacrificed and spleens were collected aseptically. Spleens were homogenized, treated with RBC lysis solution and a single mononuclear cell suspension was prepared in RPMI 1640, 1% L-Glutamine and 10% FBS.

The cells were counted and seeded in 24-well plates, with a seeding density of 3×10^6 cells/500µl/well. Then, 500 µL of five increasing BC concentrations (5, 10, 100, 1000 and 5000 µg mL⁻¹) were added to the different wells. No BC was added to the control wells. All wells were run in duplicates. Wells were then incubated for 48 h at 37°C in a 5% CO₂ incubator. After incubation, viable and dead cell count was determined.

Table 1. Protocol used in treating different groups of mice

Challenge with B16F10 melanoma cells 3.4 million cells/kg in 0.5mL					
Day 0	Untreated	Sterile water	IP BC ^a	Oral BC ^b	IP and Oral BC ^c
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

^a: Intraperitoneal injection (IP); Sterile water (0.4 mL/mouse), Bovine cartilage (75 mg mL⁻¹)

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

^c: Bovine cartilage IP (37.5 mg mL⁻¹) and through drinking water (18.5 mg/mouse)

Statistical Analysis

Whenever applicable, data were expressed as Mean ± 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 Graph pad online software. Results were considered to be statistically significant at P value <0.05.

Results

In vivo Results

VEGF Serum Levels

When compared to the untreated group, significant decrease in serum VEGF levels at 2, 4 and 6 h post-last BC treatment (p-value = 0.0008, 0.0014 and 0.0002, respectively) was seen in oral BC group. Again, when comparing IP BC group, to the sterile water group, significant decrease in serum VEGF levels was seen at 4 and 6 h after the last BC treatment (p-value = 0.0148 and 0.0014, respectively). Finally when comparing the IP and oral BC group to the sterile water group, significant decrease in serum VEGF levels was seen only at 6 h post BC treatment (p-value = 0.0006) (Fig. 1). In the sterile water group and the IP and oral group, VEGF levels at 2 h after the last BC dose were out of range (higher than the highest standard's absorbance). It is worth noting that this experiment was repeated twice using different dilution factors and consistent results were obtained.

IL-12 Serum Levels

The serum levels of IL-12, at 2 h post BC treatment, were lower in IP BC and oral BC groups, compared to

their respective control, sterile water and untreated groups; however IL-12 level of the IP and oral BC group, was equal to that of its control, sterile water group. At 4 h post-treatment, the levels of IL-12 significantly increased in IP BC group (P value = 0.003) and oral BC group (P value = 0.0330) when compared to their respective controls. However in the IP and oral BC group, a notable yet insignificant increase was seen. Finally, at 6 h post treatment, the IL-12 levels of IP BC and oral BC groups were lower than that of their respective controls, yet a significant increase in the serum level of IL-12 was seen in IP and oral BC group (P value = 0.0477) (Fig. 2).

Mice Survival

By day 20, 9 out of 10 mice were dead in IP BC group and oral BC group (10% survival rate in both groups); however 8 out of 10 mice were dead in IP and oral BC group (20% survival rate), yet none of the mice in the untreated and sterile water groups survived beyond day 19 (0% survival) (Fig. 3).

The survival results were further evaluated by generating the Kaplan-Meier survival curves showing the probability of survival in a given period of time. The statistical significance of the results obtained was assessed by determining the p-value; p-value ≤ 0.05 was considered statistically significant. The IP BC group and the oral BC group did not show significant survival results when compared to the sterile water and untreated groups, respectively. However, IP and oral BC group showed statistically significant survival results (P value = 0.047) when compared to its control, sterile water group (Fig. 4).

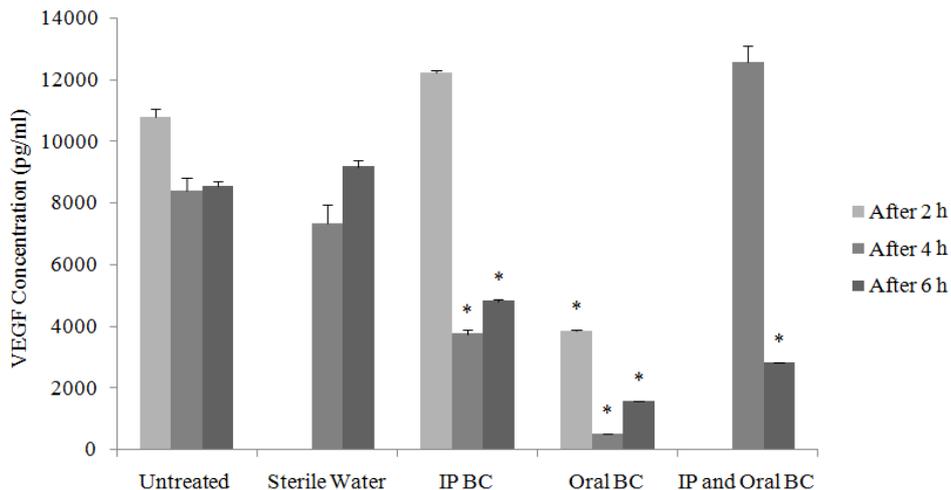


Fig. 1. Serum VEGF levels as detected by ELISA. VEGF levels decreased significantly in all groups that received BC treatment whether IP, orally or both *: Statistically significant at p-value < 0.05

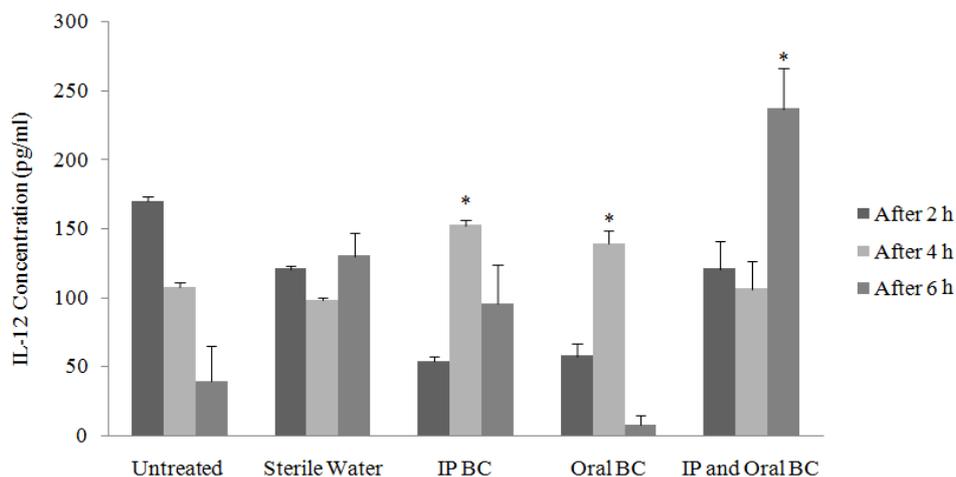


Fig. 2. Serum IL-12 levels as detected by ELISA. Significant increase in IL-12 levels was seen in IP BC and oral BC groups at 4 h post treatment and in IP and oral BC group at 6 h post treatment *: Statistically significant at p-value < 0.05

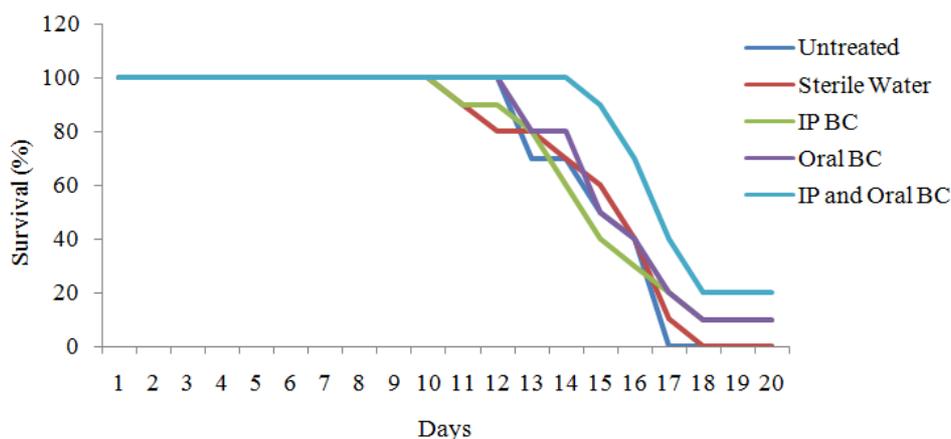


Fig. 3. Survival curve of the 5 groups of mice followed over a period of 20 days. IP and oral BC group had the highest survival rate (20%) beyond day 19

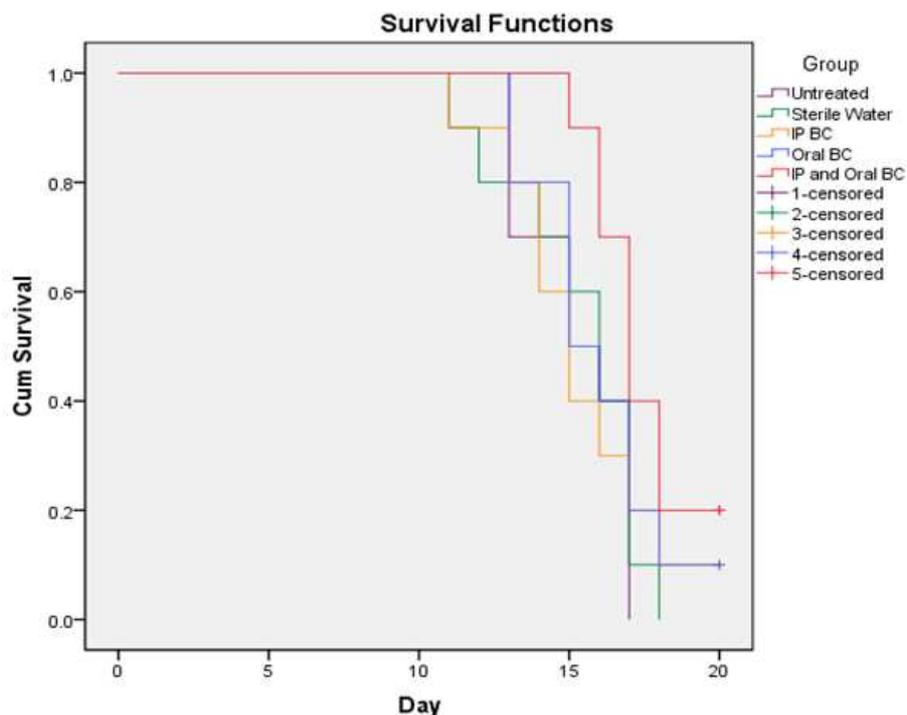


Fig. 4. Kaplan Meier survival curves for the five groups. IP and oral BC group showed statistical significant survival rate when compared to its control, sterile water treated group

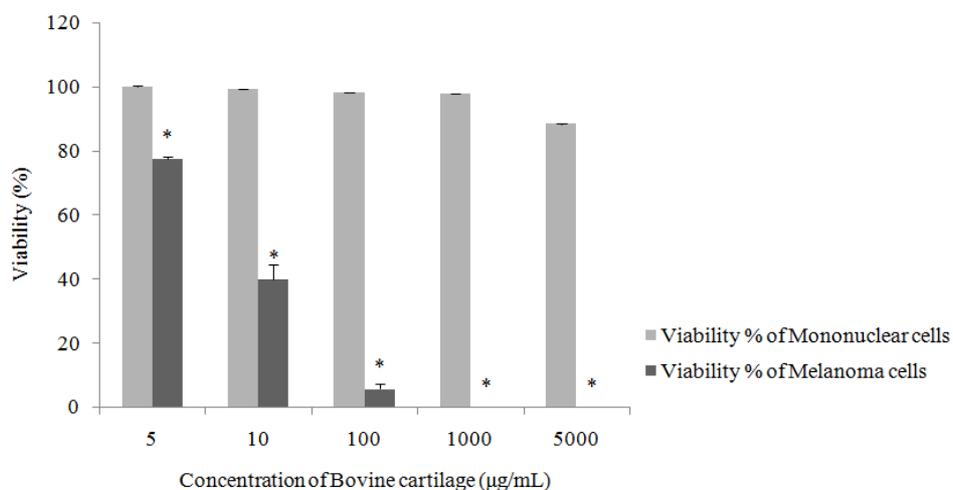


Fig. 5. Assessing the effect of BC on the growth of mouse mononuclear and melanoma cells *in vitro*. The viability of B16F10 melanoma cells significantly decreased with increasing concentration of BC, reaching a total eradication of cells at 1000 µg mL. BC had no significant effect on mouse mononuclear cells survival *: Statistically significant at p-value < 0.05

Table 2. Correlation coefficient between survival ratio and VEGF, as well as IL-12 in BC-treated mice

		IL-12		VEGF	
		-----		-----	
		Pearson's	p-value	Pearson's	p-value
		Correlation coefficient		Correlation coefficient	
Survival	2 h	-0.397	0.507	-	-
	4 h	0.267	0.663	0.218	0.724
	6 h	0.567	0.318	-0.839	0.0750

Nonetheless, the remaining one mouse from IP BC group showed complete response to BC treatment (probable cure). This mouse did not show any solid tumor growth externally unlike the remaining mice challenged with B16F10 melanoma cells. Moreover, upon dissection no necrotic organs were visible. A blood smear from the dissected mouse was prepared with trypan blue, and no large cells similar to those seen in the previous cases were observed under the microscope. These observations further led to the conclusion that this mouse might have undergone probable cure.

The linear relations between mice survival and VEGF as well as IL-12 levels were examined. The relation between the survival ratio and VEGF levels approached linearity at 6 h post BC treatment (Pearson's Correlation coefficient = -0.839, p-value = 0.0750), which may indicate that VEGF levels tend to decrease in groups with better survival (Table 2).

In Vitro Results

Viability of B16F10 Melanoma Cells Incubated with BC

After 48 h of incubation, significant decrease in viable melanoma cell count was obtained in the wells treated with 500 μL of 5 $\mu\text{g mL}^{-1}$ (P value = 0.0003), 10 $\mu\text{g mL}^{-1}$ (P value = 0.003) and 100 $\mu\text{g mL}^{-1}$ (P value = 0.0001) of BC, when compared to the control wells. Total eradication of melanoma cells was obtained in the wells treated with 500 μL of 1000 and 5000 $\mu\text{g mL}^{-1}$ of BC (Fig. 5). The *in vitro* experiment conducted on the viability of B16F10 melanoma cells was repeated three times and consistent results were obtained.

Viability of Mouse Mononuclear Cells Incubated with BC

When compared to the control well, the viability of mouse mononuclear cells remained relatively stable throughout the first four BC concentrations (5, 10, 100 and 1000 $\mu\text{g mL}^{-1}$) after 48 h; however a slight insignificant decrease was seen with the highest BC concentration which is 5000 $\mu\text{g mL}^{-1}$ (88% survival rate) (Fig. 5).

Discussion

The anti-inflammatory effect of BC and its ability to cure a patient's ulcerated breast cancer drove Prudden to assess the cartilage's effect in the clinical treatment of cancer (Prudden and Balassa, 1974; Morell and Daniel, 2014).

Although promising results were obtained, most scientists chose not to further investigate the anti-tumor activity of BC. For this purpose this study was carried out, in an attempt to determine the effect of BC on tumor

growth *in vivo*, on serum levels of the pro-angiogenic cytokine VEGF and the anti-tumor cytokine IL-12 in tumor-bearing mice and on the survival of mouse melanoma and mononuclear cells *in vitro*.

In an attempt to test whether BC acts selectively on tumor cells, B16F10 melanoma cells and mouse mononuclear cells were separately incubated with increasing concentrations of BC for 48 h. It was observed that the viability of melanoma cells decreased when treated with BC and this decrease was further enhanced with time of exposure and amount of BC added, reaching a total eradication of tumor cells at 1000 $\mu\text{g mL}^{-1}$ of BC after 48 h. In contrast, when mouse MNCs were incubated with increasing concentrations of BC, only a minute decrease in the viability was observed at 5000 $\mu\text{g mL}^{-1}$ after 48 h; indicating that even at higher doses of BC MNCs remained viable. These results were consistent with (Durie *et al.*, 1985) who demonstrated the anti-proliferative activity of BC against several tumor cell lines using the same BC concentrations. Additionally, the obtained result might further emphasize the function of BC as a strong biological modifier because it appeared that this toxicity was selective to tumor cells. Moreover, to further test this hypothesis of selective toxicity of BC, its activity *in vivo* was tested. Results showed that after receiving IP BC injections for 10 days consecutively, mice didn't show any weight, skin or hair loss for 3 months of observation; all of which are markers of toxicity. Hence, the observed results *in-vitro* and *in-vivo*, concurred with the hypothesis showing that BC has a selective toxicity against tumor cells and were also consistent with Prudden's result, who reported that BC presents no record of toxicity in animals and humans (Prudden, 1985).

When the anti-angiogenic activity of BC was evaluated *in vivo*, significant decrease in VEGF levels was seen in IP BC and oral BC groups and in the IP and oral BC group, however the significant decrease was delayed in the latter. The outcome of this experiment indicates that independent of the route of administration, BC causes a decrease in VEGF levels which is consistent with 2 previous studies; (Folkman *et al.*, 1971) who demonstrated that an inhibitor secreted from BC was able to directly inhibit tumor capillary proliferation and (Langer *et al.*, 1976) who purified a fraction from BC with a molecular weight of 16,000, demonstrated that it possesses the ability to inhibit cancer induced neovascularization. It is worth to note that, the reason behind the delayed but significant decrease of VEGF levels in IP and oral BC group might be due to the decreased BC administration dose divided between oral and IP.

The stimulatory effect of BC on Antigen Processing/Presenting cells was determined by quantifying the serum levels of Interleukin-12 (IL-12).

IL-12 possesses strong anti-tumor and anti-metastatic properties and it is mainly secreted by the Antigen Presenting Cells (APC) including macrophages (Zeh III *et al.*, 1993; Valerie *et al.*, 1993; Gately *et al.*, 1994). The ability of BC to activate macrophages was reported (Prudden, 1985; Durie *et al.*, 1985; Rosen *et al.*, 1998; Morell and Daniel, 2014). In this context, our results indicated activation of macrophages indirectly through the significantly increased levels of IL-12 seen at 4 h post-BC treatment in IP BC and oral BC groups and at 6 h post-BC treatment in IP and oral BC group. The delayed increase of IL-12 levels seen in the sera of the treated groups might be explained by the fact that BC needed more time to exert its stimulatory effect on the immune system. Moreover the decreased IL-12 levels seen in the sera of IP BC and oral BC groups at 6 h post treatment might be due to what is known as the oscillating effect of a biological response after administration of the drug. The oscillating effect is when a cycle of decrease and increase in physiological responses are seen to a given drug or substance after administration. The response doesn't remain low or high it represents as a transient cycle (Rando *et al.*, 2010).

As for the survival results, IP BC group and oral BC group did not show significant survival results when compared to their respective controls (10% survival) as opposed to the IP and oral BC group which showed significant survival rate (20%) compared to its control group. These results were consistent with the report of (Romano *et al.*, 1985) 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 (Prudden, 1985). According to (Prudden, 1985), the maintenance dosage is given orally; however, every treatment should start with injections which are regarded as a "loading phase". The loading phase is considered complete when a total of 2000 mL of cartilage is injected into the patient. This might also explain why mice in IP BC group and oral BC group which showed significant decrease in serum levels of VEGF did not show significant survival rates.

The mouse from IP BC group which might have shown probable cure even after discontinuing the treatment is similar to one of Prudden's patients who was completely cured (Prudden, 1985). Additionally, the case of this mouse resembles the situation where some cancer patients respond to cancer 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 (Poutahidis *et al.*, 2014; Sivan *et al.*, 2015).

Conclusion

It appears that BC possesses selective anti-tumor activity. This activity seems to give better results when both routes of administration of BC are utilized as suggested by Prudden. Moreover, improved results could be achieved by increasing the population number, broadening the number of cytokines quantified and using more accurate oral administration methods.

Acknowledgement

This study was partially funded by a Lebanese National Council for Scientific Research grant awarded to Dr. Alexander M. Abdelnoor

Authors' Contributions

Arax Tanelian and Dalal F. Jaber: Contributed to the conception and design of this study, acquisition of laboratory data, data analysis and/or interpretation, drafting and/or critical revision of the manuscript and approved this final version.

Nayla S. Al Akl: Participated in the data analysis and/or interpretation, drafting and/or critical revision of the manuscript and approved this final version.

Alexander M. Abdelnoor: Contributed to the conception and design of this study, drafting and/or critical revision of the manuscript and approved this final version.

Conflict of Interest

The authors declare that they have no conflict of interests.

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