# Antiproliferative effect of berberine on canine mammary gland cancer cell culture

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**Abstract.** Canine mammary gland tumors are the most frequent cause of cancer in female dogs. Numerous studies using cancer cell lines and clinical trials have indicated that various natural products and antioxidants reduce or possibly prevent the development of cancer. Berberine (BBR), the most important alkaloid in the Berberidaceae, which exerts a wide range of pharmacological and biochemical effects, has drawn much attention due to its particularly high antitumor activity *in vitro* and in animal studies. The aim of the present study was to investigate the antiproliferative effect of BBR against a canine mammary gland carcinoma cell line (CF41.Mg) *in vitro*. CF41.Mg cells were cultured in RPMI-1640 medium containing 10% heat inactived fetal bovine serum (FBS) and 100 mg/ml peniciline-streptomycin. Subsequently the cells were treated with different concentrations of BBR chloride (10, 25, 50, 100 and 200 $\mu$M) at a density of 12,000 cells/well in 96-well plates. Following treatment, the MTT assay was used to detect cell viability after 24-, 48- and 72-h incubations at 37˚C with 5% $CO_2$. The results indicated that BBR inhibited proliferation of canine mammary gland carcinoma cells, as treatment with 100 $\mu$M BBR for 24 h resulted in a significant decrease in cell viability ($P<0.005$). As the present study demonstrated that BBR (10-200 $\mu$M) induced cancer cell death, it is proposed that BBR may serve as a candidate agent against canine mammary tumor cells via its antiproliferative activity.

## Introduction

Multicellular organisms are an aggregation of cells that are responsible for particular tasks (1). The complexity of various different cell types coming together in multicellular organisms requires stringently regulated processes for cell division, proliferation and differentiation, which are controlled by numerous signaling pathways. The rate of cell division is controlled by apoptosis and differentiation (2), with the uncontrolled and unlimited growth of cells leading to cancer. Cell proliferation results in tumor formation, which may spread to distant sites by metastasis (3).

Canine mammary tumors (CMTs) are the most common type of tumor, which may affect female dogs. The prevalence of this tumor type in dogs is three times more than in humans. Today, CMT comprises 50% of all tumors that affect female dogs, and 41-53% of these appear to be malignant (4).

The initiation basis of all neoplasms is a lack of response by natural growth inhibitors, as well as a lack of control of the cell cycle. Cell death becomes a particularly vital mechanism in preserving homeostasis and contributes to preventing cancer pathogenesis. In addition to principle medical oncology therapy, a number of substitute therapeutic strategies are being applied to cancer cases. Factors generating apoptosis in cancer cells or sensitizing the cells to routine cell-death mechanisms are used as cancer therapeutics. Certain studies have reported adverse effects of herbal supplements in different types of cancer (5,6). Herbal medicine has long been administered to treat malignancies in Asian countries (7). The results of studies using different cancer cell types propose that inhibition of the cell cycle and growth are ordinary actions of phenolic phytochemicals, determined by reduced cell viability and incidence of cell cycle arrest (8). Various studies have demonstrated that extracts from a number of herbal plants exhibit anticancer activities *in vitro* and *in vivo* (6-11).

*Berberis vulgaris* L. is a widely known plant, with a history in traditional herbal medicine, which contains a large quantity of berberine (BBR), an isoquinolone alkaloid. BBR is a quaternary ammonium salt isolated from a variety of herbs, including Coptidis rhizome (12). BBR exerts various pharmacological and biochemical effects, such as anti-diarrheal, anti-arrhythmic and anti-inflammatory activities (13), and has become a point of interest due to its particularly high antitumor activity *in vitro* and *in vivo*.

As natural products of medicinal plants are considered to be significant in controlling tumors and, as the role of BBR in animal tumors is unclear, the current study examines the effect of BBR (an isoquinolone alkaloid), as a phytochemical

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anticancer agent, against proliferation of canine mammary tumor cells (CF41.Mg) *in vitro*.

## Materials and methods

*Cell culture and chemicals*. The canine mammary gland cancer cell line, CF41.Mg (ATCC® CRL-6232™) was purchased from Pasteur Institute of Iran (Tehran, Iran), and Sigma-Aldrich BBR chloride was obtained from Merck Millipore (Darmstadt, Germany).

CF41.Mg cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 100 mg/ml penicillin-streptomycin. The cells were maintained in an incubator with a humidified atmosphere of 5% $CO_2$ at 37˚C.

*Cell counting*. The cells were counted using a hemocytometer. The viability and growth of cells were estimated by direct counting of 0.4% trypan blue dye-excluding cells under a Nikon Eclipse Ti-S inverted microscope (Nikon Corporation, Tokyo, Japan).

*Cell harvesting and treatment*. Subsequently, the cells at the third passage were seeded into 96-well plates at a density of 12,000 cells/well. After one day of incubation for attaching the cells, the phenol red-free media with 10% FBS and different concentrations (10, 25, 50, 100 and 200 $\mu$M) of BBR chloride were added to the cells. In all treatments, BBR was dissolved in 0.1% dimethyl sulfoxide (DMSO) and made up to a maximum final concentration of 0.05% (v/v) in the complete cell culture medium, so as not to affect cell growth. Separate control (RPMI medium with 0.05% DMSO and cells), and blank (only medium) wells were prepared. After BBR was added, the 96-well plates were incubated for 24, 48 and 72 h at 37˚C in an atmosphere of 5% $CO_2$.

*Cell viability assay*. Following treatment, the MTT assay was used to detect cell viability. Briefly, 10 $\mu$M MTT (Thermo Fisher Scientific, Inc.; at 5 mg/ml) was added to each well, at a final concentration of 200 $\mu$g/ml and incubated for 4 h. The resulting intracellular purple formazan was solubilized and quantified by spectrophotometry. The absorbance of the sample was read at a wavelength of 570 nm and cell viability was calculated according to the following equation:

Cell viability (%) = mean absorbance of sample/mean absorbance of control x 100 and activity (%) = 100 - cell viability (%).

*Statistical analysis*. All of the experiments were repeated three times and one-way analysis of variance followed by Dunnett's test were used for data analysis. Data are presented as the mean ± standard deviation of the mean and $P<0.005$ was considered to indicate a statistically significant difference.

## Results

The growth of CF41.Mg cells treated with BBR at concentrations ranging from 10 to 200 $\mu$M was monitored within 72 h of culturing. To determine the cell viability following exposure to different concentrations of BBR in the CF41.Mg cell line, the MTT assay was performed. As shown in Figs. 1-3, the greatest inhibition of cell proliferation was observed after 24- (Fig. 1), followed by 72- (Fig. 3) and 48-h (Fig. 2) exposures at concentrations of 200 and 100 $\mu$M, while no inhibition activity was observed in the control group. The results indicate that BBR at concentrations of 100 and 200 $\mu$M had the highest antiproliferative effect after 24 h (Fig. 1). Following a 48-h exposure to different concentrations of BBR a delayed cytotoxic effect was induced (Fig. 2). Data showed a significant reduction of CMT cells that were treated with 100 and 200 $\mu$M BBR in comparison to the control group after 72 h of exposure ($P<0.005$; Fig. 3).

Briefly, the results indicated that BBR inhibited the proliferation of canine mammary gland carcinoma cells, and that treatment with 100 $\mu$M BBR for 24 h resulted in a significant decrease in cell viability ($P<0.005$; Fig. 4).

## Discussion

Cell homeostasis is regulated by various mechanisms, including proliferation, growth arrest and apoptosis. Disturbance of the equilibrium between cell growth and death often leads to carcinogenesis (14). Cell proliferation is regulated by the cell cycle, which is an involved sequence of cell growth and replication (15).

Cancer is more exactly associated with being the product of a breakdown in cell cycle regulation, such that injured or mutated cells (that are usually destroyed) are permitted to develop through the cell cycle accumulating mutations. Throughout this process, the cells are tending towards genetic injuries. Apoptosis is critical in the pathogenesis of human disease, particularly in malignancies, while the factors controlling apoptotic progression are suppressed, overexpressed or their function (mutation, phosphorylation or acetylation) is modified (16,17). Deficiency in its pathways promotes cancer cell survival and confers resistance to antineoplastic therapeutic agents. The number of studies regarding apoptosis is rapidly increasing, which has led to the possibility of novel therapeutic approaches for certain human diseases (18).

Many potential cancer-protective agents are broadly categorized as blocking agents, which impede the initiation stage, or suppressing agents, which arrest the promotion and progression of tumors, presumably by affecting or disturbing crucial factors that control cell proliferation (19). Numerous studies in previous years have demonstrated the anticancer activity of BBR against human lymphoma, leukaemia, choriocarcinoma, melanoma, and breast, skin and prostatic cancers (20).

In the present *in vitro* study, it was indicated that BBR (10-200 $\mu$M) decreased the number of CF41.Mg cells harvested, as evidenced by reductions in the cell viability. These reductions, subsequent to 24-, 48- and 72-h exposure to BBR, may be due to the inhibition of cell proliferation rather than to the cytotoxicity of the compound used. After 24 h, the highest tested concentrations (100 and 200 $\mu$M) demonstrated the greatest antiproliferative effect, which was manifested by partial degeneration of the CF41.Mg cell populations. BBR at different concentrations, after 48 h, induced a delayed cytotoxic effect. The inhibitory effect of BBR on the growth of CF41.Mg cells was markedly greater after 72 h when compared with that

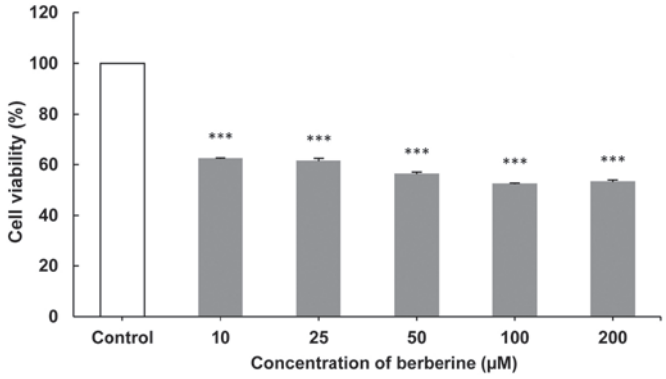


Figure 1. Effect of berberine (10, 25, 50, 100 and 200 $\mu$M) on cell viability of CF41.Mg cells using MTT assay after a 24-h exposure to berberine. ***P<0.005 vs. untreated control group.

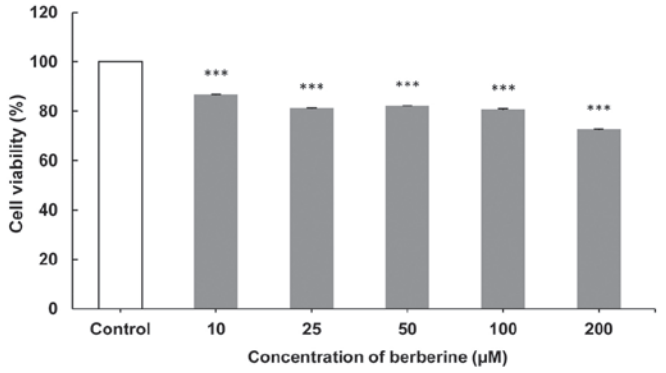


Figure 2. Effect of berberine (10, 25, 50, 100 and 200 $\mu$M) on cell viability of CF41.Mg cells using MTT assay after a 48-h exposure to berberine. ***P<0.005 vs. untreated control group.

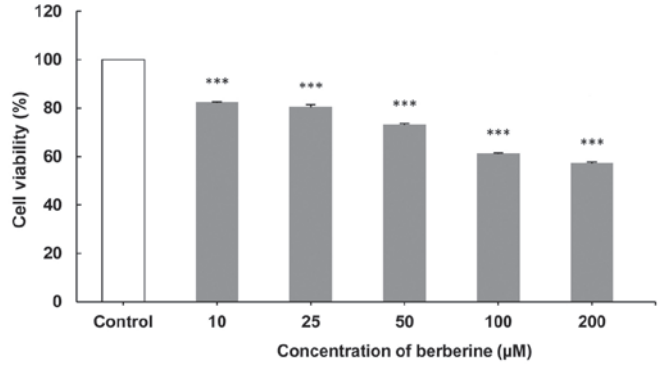


Figure 3. Effect of berberine (10, 25, 50, 100 and 200 $\mu$M) on cell viability of CF41.Mg cells using MTT assay after a 72-h exposure to berberine. ***P<0.005 vs. untreated control group.

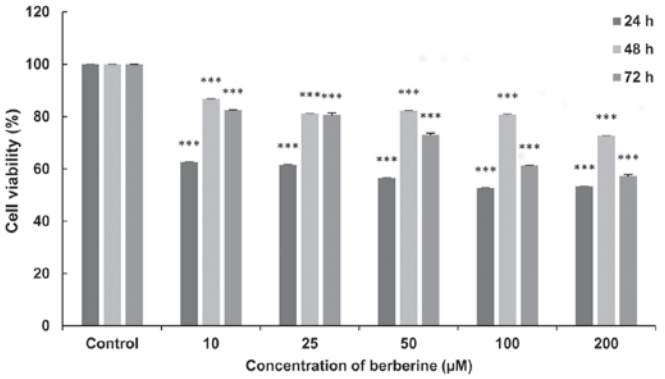


Figure 4. Effect of berberine on proliferation of CF41.Mg cells after exposure to berberine for 24, 48 and 72 h. ***P<0.005 vs. untreated control group.

at 48 h. Briefly, in Fig. 4, BBR prevented the proliferation of a markedly higher number of cells in a concentration- (but not time-) dependent manner in the exposed cells.

Previous studies indicated that BBR may lead to cell death, and hence delay cell proliferation (19,21). In addition, detailed investigations on molecular carcinogenesis provided the potential for therapeutic intervention in cancer by specifically targeting and sensitizing cancer cells to apoptosis (22,23).

Herbal remedies, such as BBR act via interactions with DNA and RNA, cell cycle arrest, and anti-inflammatory and anti-angiogenesis activities (24). The findings of the current study indicated that BBR may be more active in the inhibition of tumor cell proliferation, although BBR shows minor cytotoxicity against normal cells. For example, in the study by Meeran *et al* (25) BBR treatment enhanced reactive oxygen species (ROS) generation in prostate cancer cells, but not in normal prostate epithelial cells, which provides some explanation as to the antioxidative effect of BBR in normal cells. Furthermore, the study by Sun *et al* (20) indicated that various cell lines display marked and varied reactivity to this particular alkaloid. For example, Letasiová *et al* (26) demonstrated that the murine melanoma B16 cell line was more sensitive to BBR treatment than the human promonocytic U937 cells (the values were 75-119 times lower). In addition, different antiproliferative effects were apparent even in the same category of tumor cells. A study by Jantova *et al* (27) indicated that the cell sensitivity to BBR, in increasing order, was as follows: B16, EAC, V79, U937, L1210, NIH 3T3 and HeLa cells.

The results of the current study may be extrapolated to human research, as CMTs are being considered as a spontaneous animal model of human breast cancer. There are many similarities between human and canine mammary cancers; the two species represent a heterogeneous group, in terms of morphology and biological behavior, and in human and canine mammary cancers similar cancer-associated pathways are activated (in instances where the two species live under similar environmental conditions) (28).

Since few therapeutic agents have been developed for the treatment of different cancers, such as mammary neoplasms in animals, and as BBR appears to be capable of treating tumor cells, the current study indicates that BBR may potentially serve as a naturally occurring compound for CMT therapy.

In conclusion, due to the antiproliferative effect of BBR against CMT cells, the present *in vitro* study proposes that BBR may be utilized as a candidate therapeutic agent for the inhibition of CMT cell proliferation. However, further investigation of the underlying biological mechanisms are required to explicate the molecular interactions between BBR and cell systems, and the potential of BBR administration in tumor cell therapeutic strategies *in vivo*.

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