

Original Article

Low concentration of caffeine inhibits cell viability, migration and invasion, and induces cell apoptosis of B16F10 melanoma cells

Anqi Li¹, Ning Wu², Haiping Zou², Bohui Zhu², Sang Xiong², Gongwei Xiao²

¹Department of Chemotherapy, Fudan University Shanghai Cancer Center, Minhang Branch, Shanghai, China;

²Department of Oncology, Shanghai Xuhui Central Hospital, Shanghai, China

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Abstract: We aimed to explore the effects of low concentration of 1, 3, 7-trimethylxanthine (caffeine) on melanoma cells, as well as the underlying mechanism. In this work, B16F10 murine melanoma cells were pre-treated with different concentrations (0, 50, 100, 200, 400 or 600 μ M) of caffeine for 24 h, 48 h, or 72 h. The cell viability was determined by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The migration and invasion assay were assessed by Transwell system. The cell apoptosis were analyzed by Annexin V-Cy5 and propidium iodide (PI) staining. The protein levels of bone morphogenetic protein (BMP) 2, BMP4, BMP7, B-cell lymphoma (Bcl-2), and Bax were measured by Western blot. The results showed that caffeine (400 or 600 μ M) pretreatment significantly reduced the cell viability at 48 h and 72 h ($P < 0.05$ or $P < 0.01$) but not at 24 h. The number of migrated and invaded cells was significantly decreased by caffeine (200, 400 or 600 μ M) ($P < 0.05$ or $P < 0.01$). Moreover, the percentages of apoptotic cells were statistically increased by 200, 400 or 600 μ M of caffeine ($P < 0.05$ or $P < 0.01$). We also observed caffeine (200, 400 or 600 μ M) significantly down-regulated the levels of BMP2 and BMP4 but not BMP7, and statistically down-regulated the ratio of Bcl-2 to Bax ($P < 0.05$ or $P < 0.01$). To conclude, low concentration of caffeine inhibits cell viability, migration and invasion, and induces cell apoptosis of B16F10 melanoma cells.

Keywords: Caffeine, melanoma, cell viability, migration and invasion, cell apoptosis

Introduction

Cutaneous malignant melanoma is one of the most serious and aggressive malignancies of the skin, with an increasing incidence and high potential for developing metastases. It typically occurs in the skin but may rarely be detected in the mouth, intestines, or eye [1]. The incidence of melanoma in the United States has been estimated to be approximately 73,870 new cases in 2015 [2]. Although the incidence is confirmed to be low in China, the incidence rates of melanoma continues to climb, with approximately 20,000 new cases occurred each year [3]. Melanoma is responsible for the majority of skin cancer related deaths. The early stage of melanoma could be cured by surgery alone, while patients with advanced melanoma show poor outcome, with the five-year survival rate being 16.6% [4]. Therefore, it is

necessary to identify non-toxic agents to prevent and treat melanoma.

Recently, coffee consumption has been shown to be associated with reductions in the total cancer incidence and has an inverse association with several types of malignancies, including melanoma [5-7]. These effects might be presumably mediated by 1, 3, 7-trimethylxanthine (caffeine). Caffeine is a natural alkaloid methylxanthine and a main component of coffee, which has shown tumor preventive activity. It has been reported that caffeine could inhibit cancer cell invasiveness and metastasis in animal experiments [8, 9]. Besides, caffeine has been shown its induction of cell apoptosis [10, 11]. However, these inhibitory effects of caffeine depend on high caffeine concentrations (≥ 1 mM), which may far exceeds biologically or physiologically applicable [12]. High concentra-

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tions of caffeine could potentially lead to various adverse health effects, such as stimulation to central nervous system, teratogenicity and mutagenicity [13]. The plasma concentration of caffeine has been recommended as 80 µg/mL (412 µM) to prevent toxicity [14]. A recent study reported that low concentration of caffeine inhibited the progression of hepatocellular carcinoma (HCC) [15]. However, little information is available regarding the effects of low concentration of caffeine on melanoma.

Therefore, the present study was aimed to explore the low concentration of caffeine on melanoma. B16F10 murine melanoma cells were exposed to different concentrations of caffeine, and then the cell viability, migration and invasion, and apoptosis were determined. Also, we investigated the underlying mechanisms with respect to migration and invasion, and apoptosis. Our study might provide new insights into the prevention and treatment melanoma.

Materials and methods

Cell culture

B16F10 murine melanoma cells (CRL6323) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). B16F10 cells were grown in Dulbecco's modified Eagle medium (DMEM) (Gibco, Life Technologies) containing 10% fetal bovine serum (FBS) (Gibco, Life Technologies), 2 mM L-glutamine (Sigma-Aldrich, St, Louis, MO, USA), 100 U/ml penicillin, 100 µg/ml streptomycin (Life Technologies), and 10 mM N-2-hydroxyethylpiperazine-NO-2-ethanesulfonic acid (HEPES) buffer (Sigma-Aldrich) at 37°C in a humidified atmosphere of 5% CO₂. Culture medium was changed every three days.

Cancer cells treatment with caffeine

Caffeine (Sigma-Aldrich) was dissolved in phosphate-buffered saline (PBS) and six different concentrations were prepared (0, 50, 100, 200, 400 or 600 µM). When the cells were 70-80% confluent, the cells were exposed to six different concentrations of caffeine in free medium. Cells exposed to 0 µM of caffeine was considered as a control group. Cell supernatant was collected after 24 h, 48 h or 72 h of exposure to different concentrations of caffeine.

Cell viability

Cell viability assay was determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as previously described [16]. Briefly, B16F10 cells were seeded in 96-well plates at a density of 3×10^3 cells/well, and treated with different concentrations of caffeine (0, 50, 100, 200, 400 or 600 µM) for 24 h, 48 h, or 72 h. MTT solution (Sigma-Aldrich) (0.5 mg/mL) was added to each well and incubated for another 4 h. Then dimethyl sulfoxide (DMSO; 150 µL; Life Technologies) were added to each well to dissolve formazan crystals. The absorbance at 570 nm was measured using an automated microplate reader (EMax Endpoint ELISA Microplate Reader, Molecular Devices).

Migration and invasion assay

After incubation with different concentrations of caffeine (0, 50, 100, 200, 400 or 600 µM) for 72 h, migration and invasion assay were carried out using Transwell system (Corning, Cambridge, USA) with 8 µm pore polycarbonate membrane. For the migration assay, the cells (5×10^4 cells/well) were placed in the upper chamber of matrigel-coated transwell filters and were allowed to migrate at 37°C. The lower chamber was filled with 1 mL DMEM. After incubation for 24 h, the non-migrated cells were scraped from the upper surface with a cotton swab, and the migrated cells on the bottom surface were fixed with methanol and stained with crystal violet (Ricca Chemical Company, Arlington, TX, USA). The number of positive cells was counted in 10 random fields at $\times 200$ magnification. For the invasion assay, the method was similar to the migration assay but without matrigel-coated transwell filters.

Apoptosis assay

Apoptosis assay was determined by Annexin V-Cy5 and propidium iodide (PI) staining (BD Biosciences). Briefly, the cells were seeded at a 2×10^5 per 35-mm culture dish and pre-treated with indicated concentrations of caffeine (0, 50, 100, 200, 400 or 600 µM) for 72 h. The cells were then re-suspended in 100 µL of Annexin V-binding buffer containing Annexin V-Cy5 and PI and incubated for 15 min in the dark at room temperature. Subsequently, the cells were analyzed by flow cytometry using a

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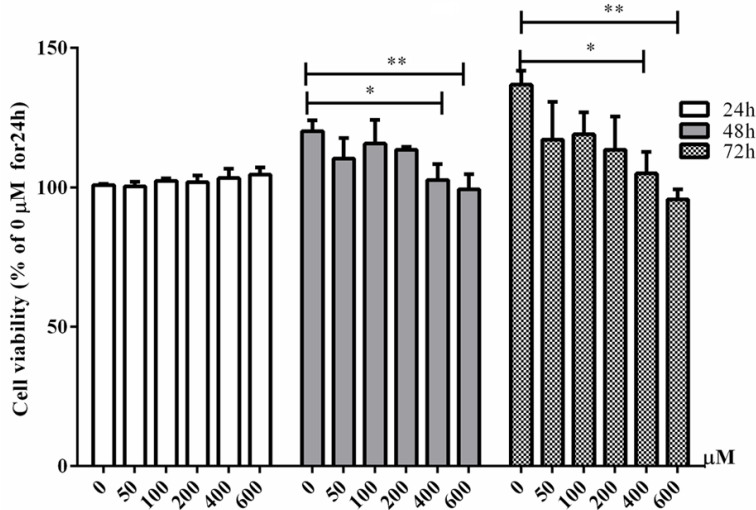


Figure 1. Effects of caffeine on cell viability. B16F10 cells were pre-treated with different concentrations (0, 50, 100, 200, 400 or 600 μM) of caffeine for 24 h, 48 h, or 72 h. The effects of caffeine on cell viability were analyzed by MTT. MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide. * $P < 0.05$ or ** $P < 0.01$ compared with control group.

FACS Calibur flow cytometer (BD Biosciences). The percentage of total apoptotic cells was expressed as early apoptosis (annexin V-Cy5 positive/PI negative) and late apoptosis (annexin V-Cy5 positive/PI positive).

Western blot

The protein levels of bone morphogenetic protein (BMP) 2, BMP4, BMP7, B-cell lymphoma (Bcl)-2, and Bax were determined by using Western blot. Briefly, the cells were treated with different concentrations of caffeine (0, 200, 400 or 600 μM) for 72 h, and cell supernatant was collected and lysed with lysis buffer containing protease and phosphatase inhibitor. The protein content was assessed using a Bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich). Equivalent amounts of protein (30 $\mu\text{g}/\text{lane}$) were subjected to 10-12% sodium dodecyl sulfonate (SDS) polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Sigma-Aldrich). The membranes were then blocked with 5% non-fat milk for 2 h and incubated with following primary antibody overnight at 4°C: anti-BMP2 antibody (ab14933, Abcam, Cambridge, USA), anti-BMP4 antibody (ab39973, Abcam, Cambridge, USA), anti-BMP7 antibody (ab56023, Abcam, Cambridge, USA), anti-Bcl-2 antibody (sc-7382, Santa Cruz, CA), and anti-Bax antibody (sc-

23959, Santa Cruz, CA). β -actin (sc-1616, Santa Cruz, CA) was used as a loading control. Subsequently, the membranes were incubated with appropriate horseradish peroxidase (HRP)-conjugated anti-goat IgG (sc-2378, Santa Cruz, CA) for 1.5 h at room temperature. The membranes were visualized by enhanced chemiluminescence (ECL) system (Pierce, USA) and analyzed using Bio-Rad Quantity One 1-D Analysis Software (Bio-Rad Laboratories).

Statistical analysis

The experiment was repeated at least three times. Data are expressed as the mean \pm standard error of mean (SEM).

Student's t test or the one-way analysis of variance (ANOVA) test was performed to analyze the data. A P -value of < 0.05 was considered as statistically significant. Results were analyzed using GraphPad Prism V6.0 (Graphpad Software San Diego, CA).

Results

Effects of caffeine on cell viability

To investigate the effects of caffeine on B16F10 murine melanoma cells, we pre-treated B16F10 cells with different concentrations (0, 50, 100, 200, 400 or 600 μM) of caffeine. After 24 h, 48 h, and 72 h of incubation, the cell viability was analyzed. As shown in **Figure 1**, the results showed that cell viability had no significant differences with different concentrations (0, 50, 100, 200, 400 or 600 μM) at 24 h compared to the control group. Besides, there were no significant differences with 50, 100, and 200 μM of caffeine on cell viability at either 48 h or 72 h. However, the cell viability was strikingly decreased by different concentrations of caffeine at either 48 h or 72 h. In addition, the inhibitive role of caffeine on cell viability was obviously under 400 or 600 μM ($P < 0.05$ or $P < 0.01$) with a dose-dependent manner compared with control cells at 48 h and 72 h. The results indicated that caffeine could inhibit the proliferation of B16F10 melanoma cells.

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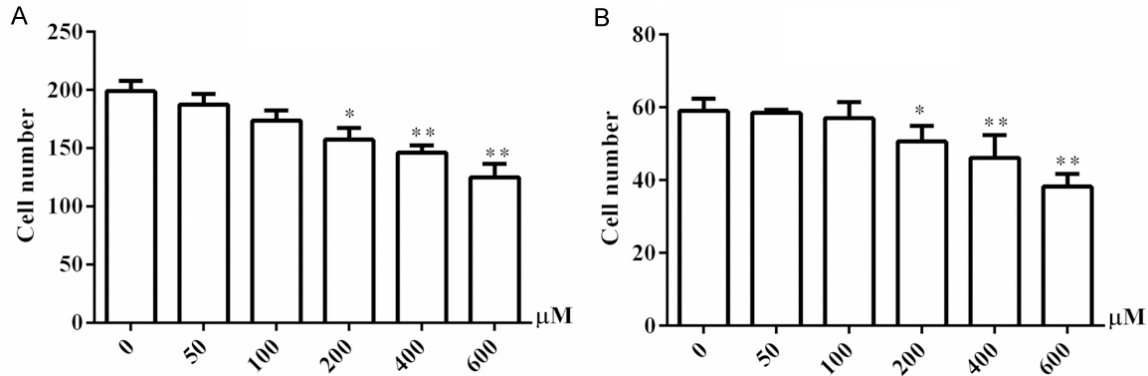


Figure 2. Effects of caffeine on migration and invasion. B16F10 cells were pre-treated with different concentrations (0, 50, 100, 200, 400 or 600 μM) of caffeine for 72 h. The effects of caffeine on migration and invasion were determined by using transwell assay. * $P < 0.05$ or ** $P < 0.01$ compared with control group.

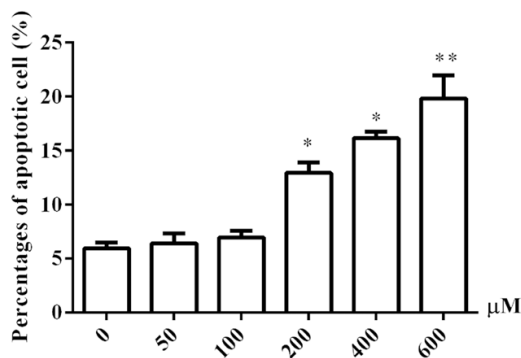


Figure 3. Effects of caffeine on cell apoptosis. B16F10 cells were pre-treated with different concentrations (0, 50, 100, 200, 400 or 600 μM) of caffeine for 72 h. The effects of caffeine on cell apoptosis were measured by using Annexin V-Cy5 and PI staining. PI, propidium iodide. * $P < 0.05$ or ** $P < 0.01$ compared with control group.

Effects of caffeine on migration and invasion

Furthermore, we evaluated the effects of caffeine on migration and invasion of B16F10 melanoma cells by using transwell assay. The results showed that the numbers of migrated cells were notably reduced by pre-treatment with different concentrations of caffeine. No obvious significant differences were found under 50 or 100 μM compared to the control group, but there were significant differences under 200, 400 or 600 μM ($P < 0.05$ or $P < 0.01$) (Figure 2A). The results of invaded numbers were similar with the results of migrated cells (Figure 2B). These data demonstrated that caffeine could inhibit the migration and invasion of B16F10 melanoma cells.

Effects of caffeine on cell apoptosis

Moreover, we analyzed the effects of caffeine on cell apoptosis. After pre-treatment with different concentrations (0, 50, 100, 200, 400 or 600 μM) of caffeine for 72 h, the cell apoptosis was determined by Annexin V-Cy5 and PI staining. As indicated in Figure 3, we observed that the cell apoptosis was statistically increased by pre-treatment with caffeine under 200, 400 or 600 μM ($P < 0.05$ or $P < 0.01$), indicating that caffeine could induce the cell apoptosis of B16F10 melanoma cells.

Effects of caffeine on expression of BMP2, BMP4, and BMP7

We further examined the molecular mechanisms of inhibitory and regulatory effects of caffeine on migration and invasion. It has been reported that BMPs are overexpressed in malignant melanoma and promotes cell invasion and migration [17]. Therefore, the protein expression levels of BMP2, BMP4, and BMP7 were measured after pre-treatment with different concentrations (0, 200, 400 or 600 μM) of caffeine for 72 h using Western blot. We found that both the protein levels of BMP2 and BMP4 were significantly reduced by pre-treatment with caffeine ($P < 0.05$ or $P < 0.01$) (Figure 4A and 4B). However, no obvious significant differences were found in protein levels of BMP7. The results indicated that caffeine could inhibit the migration and invasion of B16F10 melanoma cells by regulating the expression of BMP2 and BMP4.

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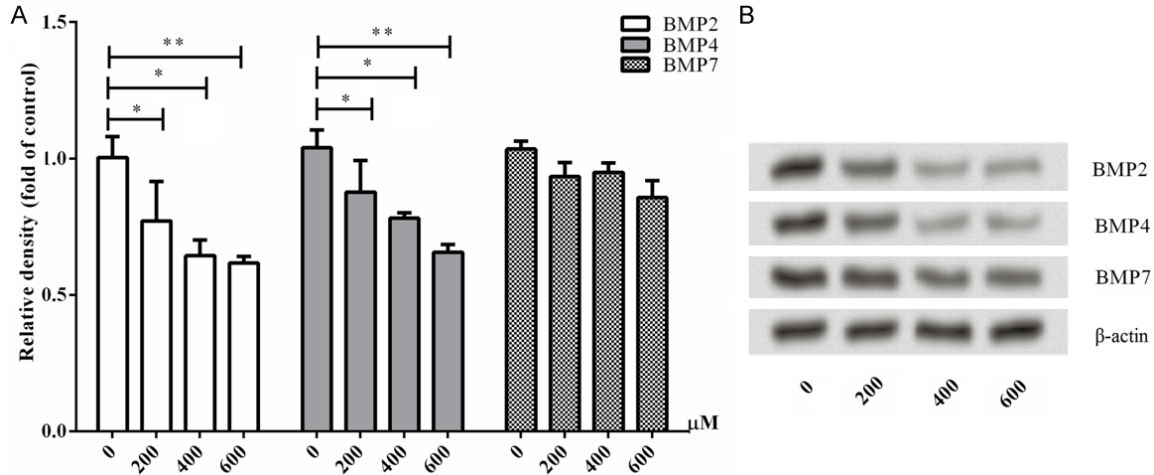


Figure 4. Effects of caffeine on expression of BMP2, BMP4, and BMP7. B16F10 cells were pre-treated with different concentrations (0, 200, 400 or 600 μM) of caffeine for 72 h. The effects of caffeine on protein expression levels of BMP2, BMP4, and BMP7 were assessed by Western blot. BMP, bone morphogenetic protein. * $P < 0.05$ or ** $P < 0.01$ compared with control group.

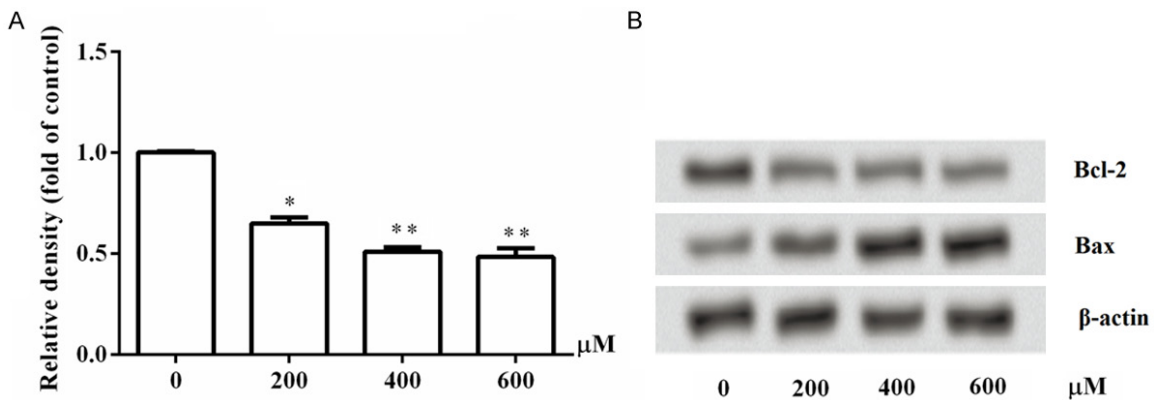


Figure 5. Effects of caffeine on expression of Bcl-2 and Bax. B16F10 cells were pre-treated with different concentrations (0, 200, 400 or 600 μM) of caffeine for 72 h. The effects of caffeine on protein expression levels of Bcl-2 and Bax were assessed by Western blot. Bcl, B-cell lymphoma. * $P < 0.05$ or ** $P < 0.01$ compared with control group.

Effects of caffeine on expression of Bcl-2 and Bax

The molecular mechanisms of regulatory effects of caffeine on induction of cell apoptosis were explored by determining the protein expression levels of Bcl-2 and Bax. As demonstrated in **Figure 5A** and **5B**, the results showed that pre-treatment with different concentrations (200, 400 or 600 μM) of caffeine significantly decreased the expression levels of Bcl-2 but increased the levels of Bax. The ratio of Bcl-2 to Bax was significantly down-regulated ($P < 0.05$ or $P < 0.01$). The data suggested that caffeine could induce cell apoptosis of B16F10

melanoma cells by decreasing the ratio of Bcl-2 to Bax.

Discussion

In the present study, we explored the effects of low concentrations of caffeine on cell proliferation, metastasis, and apoptosis of B16F10 melanoma cells, as well as possible underlying mechanism. Our results proved that caffeine could inhibit the proliferation and metastasis, and induce the apoptosis of B16F10 melanoma cells at physiologically applicable concentration. The inhibitory metastasis induced by caffeine might be by regulating the expression

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of BMP2 and BMP4, while the induction of cell apoptosis might be via regulating the expression of Bcl-2 and Bax.

Caffeine is a widely consumed stimulant and enjoyed by people throughout the world for many years [18]. It can be found in many beverages and food, such as coffee, tea, chocolate, and energy drinks. It has been well demonstrated that caffeine shows anticancer effects in various types of tumors. Many mechanisms have been reported to be responsible for the anticancer function [19, 20]. For example, caffeine has been reported to disturb cell cycle function and induce apoptosis [21]. However, most studies confirmed high concentrations of caffeine exerted the anticancer activity. Jang *et al.* found that caffeine induced apoptosis in human neuroblastoma cells at 10 mM [22], Qi *et al.* suggested that caffeine induced TP53-independent G(1)-phase arrest and apoptosis in human lung tumor cells at 5 mM [23], and Wang *et al.* observed that caffeine could increase radiosensitization to orthotopic transplant in LM3 cells at 2.5 mM [24]. However, it has been estimated that over 100 cups of coffee intake might reach a 2 mM blood level [25], which far exceeds the limitation of physiologically applicable concentration (80 µg/mL or 412 µM) [14]. Therefore, high concentrations of caffeine should be avoided to prevent the undesirable side effects. But recently, many studies have focused on the effects of low concentration of caffeine on cancers. Kawano *et al.* found that 500 µM of caffeine could enhance the anticancer effects of Cisplatin [26]. Liu *et al.* suggested that 100 µM of caffeine could decrease the invasion of human leukemia U937 cells [27]. Dong *et al.* observed that 400 or 600 µM of caffeine inhibited the proliferation, and 200, 400 or 600 µM of caffeine hindered migration and invasion *in vitro* [15]. On the basis of these data, we therefore speculated that low concentrations of caffeine might be beneficial for melanoma. To confirm the speculation, B16F10 melanoma cells were subjected to different concentrations of caffeine and then the effects of caffeine on cell viability, migration and invasion, and apoptosis were investigated. Similarly, our data showed that application of caffeine inhibited the proliferation at 48 h and 72 h, but had no effects on cell proliferation at 24 h. Besides, the anti-proliferation effects showed a dose manner with the time

increasing. Moreover, we observed that 200, 400 or 600 µM of caffeine significantly down-regulated the numbers of migrated and invaded cells and statistically up-regulated the percentages of apoptotic cells.

We further explored the anti-metastasis and pro-apoptotic effects of caffeine. Cell migration and invasion have been considered as one of the most important process in the development of cancer, leading to morbidity and mortality [28]. An emerging body of evidence has suggests that BMP family members are involved in many types of cancers, including malignant melanoma [17, 29, 30]. BMPs are extracellular signaling molecules that are the members of transforming growth factor (TGF) β superfamily [31]. Overexpressed BMP2, BMP4, and BMP7 have been reported in malignant melanoma and promote melanoma cell invasion and migration and play significant roles in the progression of malignant melanoma [17]. Our results showed that the expression levels of BMP2 and BMP4 were significantly decreased by administration of caffeine (200, 400 or 600 µM), but the levels of BMP7 were not significantly changed. The data indicated that caffeine inhibited the migration and invasion of B16F10 melanoma cells by reducing the expression of BMP2 and BMP4 but not BMP7. Apoptosis is another important pathological change that is responsible for cancers [32]. Bcl-2 family plays significant roles in the control of apoptosis, which has a potentially therapeutic role in the treatment of cancer. Bcl-2 is a critical apoptosis-inhibiting gene, and while Bax is an important apoptosis-accelerating gene [33]. The ratio of Bcl-2 to Bax determines survival or death to an apoptotic stimulus. Our results showed that administration of caffeine (200, 400 or 600 µM) markedly decreased the ratio of Bcl-2 to Bax, indicating that caffeine significantly induced cell apoptosis.

In conclusion, we find that low concentration of caffeine inhibits cell viability, migration and invasion, and induces cell apoptosis of B16F10 melanoma cells. Caffeine might be a potential, safe, effective candidate for treatment of melanoma at physiologically applicable concentration.

Disclosure of conflict of interest

None.

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Address correspondence to: Dr. Ning Wu, Department of Oncology, Shanghai Xuhui Central Hospital, No. 966, Huaihai Middle Road, Xuhui District, Shanghai 200031, China. E-mail: wuning303@126.com

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