Original Article

The Inhibitory Effects of Vanillin on the Growth of Melanoma by Reducing Nuclear Factor-κB Activation

Marjan Pourhadi[1], Ahmad Ghasemi[1], Reza Abediny[1], Shaghayegh Haghjooy Javanmard[2], Golnaz Vaseghi[1,3]

[1]Applied Physiology Research Center, Cardiovascular Research Institute, Isfahan University of Medical Sciences, [2]Department of Physiology, Applied Physiology Research Center, School of Medicine, Cardiovascular Research Institute, Isfahan University of Medical Sciences, [3]Isfahan Cardiovascular Research Center, Cardiovascular Research Institute, Isfahan University of Medical Sciences, Isfahan, Iran

Abstract

Background: Melanoma is skin cancer, and the treatments are not efficient enough. Therefore, finding new drugs seems to be an essential need. Vanillin, which is extracted from vanilla seed, has anti-cancer effects by reducing nuclear factor-κB (NF). We explored the anti-tumor effects of vanillin in the melanoma model and its possible mechanism. **Materials and Methods:** In the MTT assay, mice melanoma cells (B16F10) were treated with vanillin (1, 2, 3, 4, 5 μg/mL) for 24 and 48 h. In an animal model, B16F10 was subcutaneously injected into C57BL/6 mice. After the development of tumors, the mice were treated with 50 and 100 mg/kg/day of vanillin for 10 days. The tumor size and expression level of NF-κB protein were measured. **Results:** In the MTT assay, vanillin in all concentrations significantly decreased B16F10 cell viability after 24 h incubation. The size of melanoma tumors was reduced in both doses 50 and 100 mg/kg/day in mice. NF-κB protein expression was decreased in the 100 mg/kg/day group in comparison with the control group. **Conclusion:** We found that vanillin by reducing NF-κB expression may have anti-tumor effects and reduced melanoma tumor size and cell viability.

Keywords: *B16F10, melanoma, nuclear factor-κB, vanillin*

Introduction

Melanoma is a type of skin cancer which is one of the most common and aggressive skin cancer with a poor prognosis and high mortality rate.[1,2] Conventional treatments, such as chemotherapy, are associated with high side effects and drug resistance,[3] and advanced therapies, such as immunotherapy, are too expensive for all people.[4] Therefore, finding new drugs can be an essential step in melanoma treatment.[5] Natural products are important sources for the design and discovery of new anti-cancer drugs.[6] Today many of the anti-cancer drugs are derived from plant products.[7]

Vanillin is one of the main compounds extracted from the vanilla bean.[8] It is commonly used in the food and pharmaceutical industry as a flavoring ingredient.[9] Vanillin has antioxidant properties and prevents DNA mutagenesis by DNA repairing. This compound has anti-cancer, antimetastatic, antiangiogenic, and cytotoxic activity[10] with low side effects. Up to relatively high concentration, no significant side effects have been observed from this natural compound.[11] A study showed that vanillin up to 300 mg/kg orally or subcutaneously did not show significant toxic effects.[12]

Probably one of the mechanisms of the anti-cancer effects of vanillin is reducing nuclear factor-κB (NF).[13] NF-κB is a translation factor that plays a role in regulating and cell proliferation in the immune system.[14] There is a relationship between cancer and NF-κB. Researchers have shown that NF-κB expression in cancer cells results in the production of anti-apoptotic proteins which leads to the survival and growth of cancer cells.[15] NF-κB dysregulation can lead to melanocyte transformation to cancer cells.[16] In human melanoma, the NF-κB expression was increased especially in metastatic melanoma.[17,18] Vanillin can reduce the expression of NF-κB,[19] so we suggest that vanillin can be used as a new drug in the treatment of melanoma. In this study, we look into the effect of vanillin on melanoma and its possible anti-cancer mechanism.

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Address for correspondence:
Dr. Golnaz Vaseghi, Applied Physiology Research Center, Cardiovascular Research Institute, Isfahan University of Medical Sciences, Isfahan, Iran. E-mail: golnazvaseghi@yahoo.com

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Materials and Methods

Cell and materials

Vanillin was taken from Sigma (San Diego, California, USA) and dissolved in normal saline and 5% dimethyl sulfoxide (DMSO). B16F10 cell line was purchased from the Pasture Institute (Tehran, Iran). Dulbecco's Modified Eagle's Medium (DMEM), antibiotics of penicillin and streptomycin, and the fetal bovine serum (FBS) were obtained from Gibco BRL (Carlsbad, CA, USA). Antibody against NF-κB was taken from Santa Cruz Biotechnology (Dallas, TX, USA).

Cell treatment

The B16F10 cells were cultured in DMEM containing 10% (v/v) FBS and antibiotics (100 IU/ml penicillin and 100 μg//ml streptomycin). The culture was maintained at 37°C in a 5% CO_2 incubator.

Cell viability assay

The effects of vanillin on B16F10 viability were measured by MTT assay. Briefly, 2×10^4 cells were placed in each well of the 96-well plate in DMEM medium for 1 day. The cells were incubated with 20 μL of vanillin in different concentrations (1, 2, 3, 4, 5 μg/mL). For each concentration, three wells were used. After 24 or 48 h treatment, 20 μL of MTT solutions was added to each well and incubate the plate at 37°C for 4 h. The crystals were dissolved in 150 μL DMSO for each well (150 μL of DMSO alone as blank), and absorbance was measured by ELISA plate reader (ELX 800-BioTek-USA) at 570 nm. The formula (1) was used to determine the viability of cells:

Formula (1)

$$\text{Percent cell viability} = \frac{\text{Mean absorption of sample wells} - \text{Mean absorption of blank wells}}{\text{Mean absorption of control wells} - \text{Mean absorption of blank wells}} \times 100$$

Experimental animals

Male C57BL6 mice aged 6–8 weeks, weighing 20–28 g, were purchased from Pasture Institute (Tehran, Iran). They were kept in the Animal Laboratory with a standard 22°C–23°C temperature. Water and foods were freely available. The animal experiments were done according to the Ethical Committee of Isfahan University of Medical Sciences Isfahan, Iran (approval ID: IR.MUI.MED.REC.1398.651 on February 23, 2020).

In vivo experimental

The B16F10 was suspended in PBS till a concentration of 1×10^6 cells/100 μl was achieved. The cell suspensions (100 μl/mouse) were subcutaneously injected into the back of mice. On day 7 after injection mice randomly were divided into three groups (6–8 mice in each group). Two groups received 50, 100 mg/kg/day vanillin respectively through intraperitoneal injections. The control group received normal saline and 5% DMSO as the vehicle. The mice were sacrificed after 10 days of treatment with vanillin, and the tumors were excluded for further studies

Tumor volume

The anti-tumor effect was investigated by measuring the weight and volume of the tumor. The Vernier calipers were used to determine the length and width of the tumor, and finally, the tumor volumes were calculated by the formula (2) as known the modified ellipsoidal formula:[20]

Formula (2)

$$\text{Tumor volume} = \frac{\text{Length} \times \text{width}^2}{2}$$

Immunohistochemical staining

Immunohistochemical (IHC) was used to determine the expression of NF-κB. Tumor tissues were fixed in 10% formalin solution for 24 h. The tissue block is embedded in paraffin, then affixed onto the slide. Slides were deparaffinized in xylene and rehydrated in ethanol gradient (100%–70%). The antigen retrieval step has been done according to the antibody datasheet. The slides were incubated with 3% H_2O_2 for 30 min. For IHC staining, the slides were washed with water and treated with the NF-κB antibody (mouse monoclonal, sc-293072 hP) overnight at 4°C. The next day, the slides were incubated by 3, 3′-diaminobenzidine, and hematoxylin staining, respectively. The slides were then photographed using a Leica camera (DFC450 C). FIJI (ImageJ) software was used to analyze the NF-κB expression.

Statistical analysis

Statistical analysis was performed one-way analysis of variance followed by *post hoc* Tukey using SPSS version 20 statistical software (SPSS Inc, IL, USA). All values have been expressed as mean ± standard error of the mean. $P < 0.05$ was considered to be statistically significant when compared to control.

Results

The effect of vanillin on B16F10 cell viability

In the MTT assay, after 24 h treatment of B16F10 cells with vanillin, cell viability significantly decreased in all concentrations ($P < 0.001$). After 48 h incubation of cell, the significant decrease of cell viability was only observed when cells were treated with concentrations of 2 ($P = 0.003$) and 5 μg/ml ($P < 0.001$) of vanillin [Figure 1].

Effect of vanillin on tumor volume and tumor weight

Tumor developing in the untreated control mice was higher than two groups receiving vanillin. After 10 days of treatment with vanillin, the average tumor

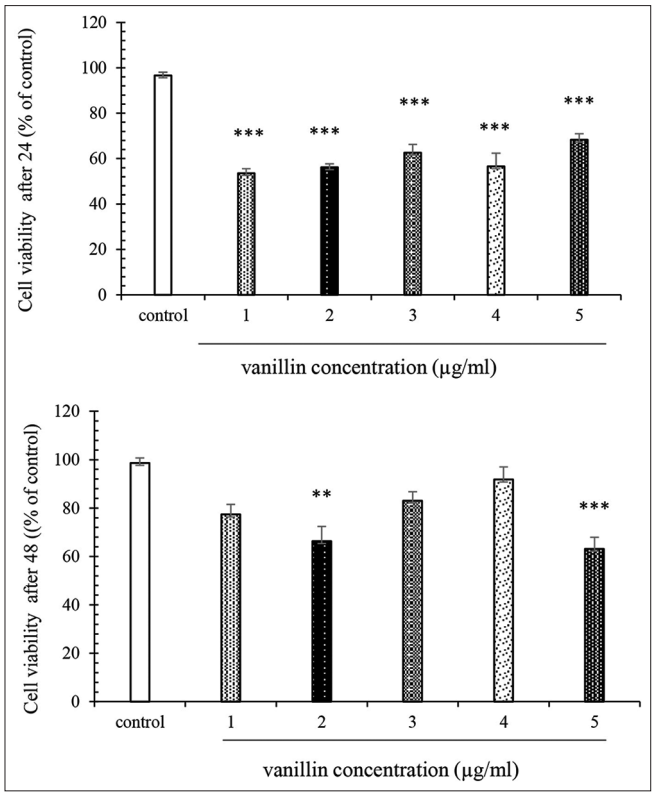

Figure 1: The effect of vanillin on B16F10 cell viability. Cells were exposed to different concentrations of vanillin for 24 and 48 h. **$P < 0.01$, and ***$P < 0.001$ and values are expressed as mean ± standard error of the mean ($n = 3$)

volume was 234.8 ± 29.633 and 442.5 ± 1.28 in 50 and 100 mg/kg/day respectively, while in the control group was 827.4 ± 91.185. Statistical analysis showed that vanillin significantly reduces the tumor volume in treated groups in both doses of 50 mg/kg/day ($P < 0.001$) and 100 mg/kg/day ($P = 0.005$) in comparison to the control group [Figure 2]. The administration of vanillin also significantly decreased tumor weight at doses of 50 mg/kg/day ($P = 0.002$) and 100 mg/kg/day ($P = 0.045$) compared to the control group [Figure 3]. The average tumor weight was 0.244 ± 0.033, 0.426 ± 0.097, and 0.7002 ± 0.067 in 50, 100 mg/kg/day, and the control group, respectively. There was no significant difference between the two groups receiving 50 and 100 mg/kg/day vanillin in tumor volume ($P = 0.125$) and tumor weight ($P = 0.208$).

Immunohistochemical evaluation of nuclear factor-κB expression

The NF-κB expression was significantly decreased in the 100 mg/kg/day group compared with the control group [$P = 0.019$; Figure 4]. Vanillin reduced NF-κB expression in the 50 mg/kg/day group, but it was not significant when compared with the control group [$P = 0.086$; Figure 4]. There was no significant reduction of NF-κB expression between 50 and 100 (mg/kg/day) groups ($P = 0.619$).

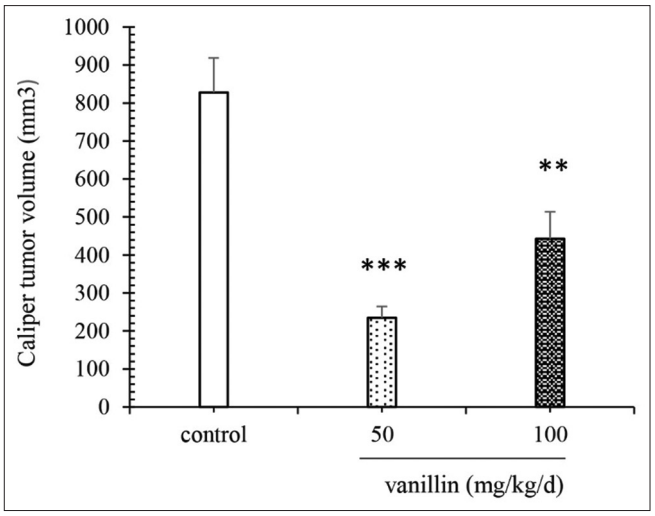

Figure 2: Mean melanoma tumors volume in different groups. Tumor-bearing animals were treated with different dose of vanillin for 10 days. **$P < 0.01$, and ***$P < 0.001$ and values are expressed as mean ± standard error of the mean ($n = 6$–8)

Discussion

In this study, we show that vanillin had anti-tumor activity both *in vivo* and *in vitro* and reduced melanoma tumor size by suppressing the NF-κB expression.

Vanillin is a natural compound with anti-neoplasm effects[21] and low adverse effects.[22] Hitherto, several studies have been done to find the anti-neoplastic effects of vanillin.[21] For example, one study has been shown vanillin could prevent breast cancer metastasis by inhibiting the enzyme activity of matrix metalloproteinases (MMPs).[11] It had synergistic effects with doxorubicin in breast cancer. Vanillin without doxorubicin also was able to reduce the tumor volume significantly compared to the control group in breast cancer.[23] The anti-cancer effects of vanillin on gastrointestinal malignancies have also been studied. It could cause death in colon cancer cells (HT-29) by inducing apoptosis, and cell cycle arrest both low (200 mg/ml) and high concentrations (1000 mg/ml). G0/G1 arrest was achieved in low concentrations and G2/M arrest in high concentrations of vanillin.[24] The reduction of cell proliferation by vanillin in melanoma has also been reported. This natural compound inhibited the proliferation of cancer cells by reducing the expression of HIF-1 in melanoma cancer cell lines (A2058 and A375).[25] Our MTT and animal test results show that vanillin could inhibit the growth of melanoma cells. However, in the MTT assay, the reduction of cell proliferation was significant in all concentrations of vanillin in 24 h but 48 h just in two concentrations. Also, in animal study, vanillin reduce the tumor volume and weight by 50 mg/kg/day more than 100 mg/kg/day. Probably that this paradox could be due to the effect of vanillin in DNA damage repair, which increased after 48 h and by increasing the dose (10).

The result suggested that vanillin suppressed melanoma tumor volume and weight. However, the precise

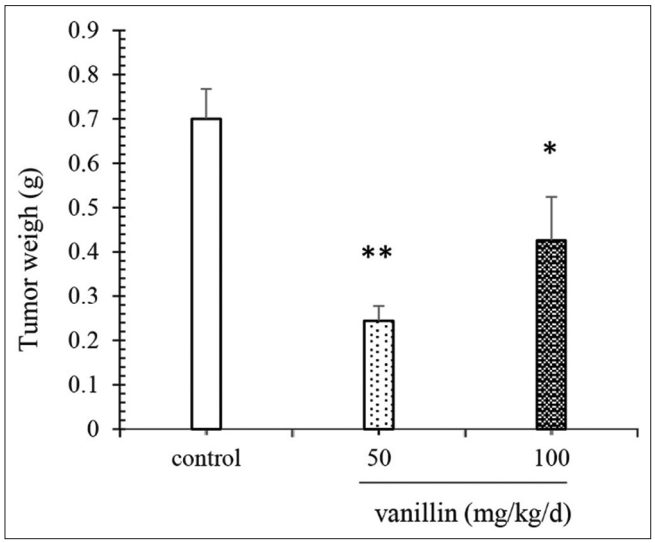

Figure 3: Mean melanoma tumors weigh in different groups. Tumor-bearing animals were treated with different dose of vanillin for 10 days. *$P < 0.05$, **$P < 0.01$ and values are expressed as mean ± standard error of the mean ($n = 6–8$)

mechanism whereby vanillin decreases tumor size is not well known.[25] In this research work, we investigated the hypothesis that vanillin could reduce melanoma tumor size by inhibiting NF-κB expression. Increased NF-κB expression has been seen in many cancers such as melanoma.[26] NF-kB activation can lead to drug and radiation resistance.[27,28]

Canonical and noncanonical are generally two main pathways for NF-κB activation. In the canonical pathway, one of the factors that activate NF-κB is the phosphorylation of p65. Therefore, any factors that prevent p65 phosphorylation can inhibit NF-κB and may have anti-cancer.[29] Various studies have been performed on the reduction of NF-κB expression by vanillin. One study has been shown vanillin inhibited p65 phosphorylation. This led to a decrease in NF-κB expression and finally increased the induction of apoptosis in cancer cells mediated by TRAIL (an anti-cancer agent).[30] These results were repeated in another study and vanillin reduced

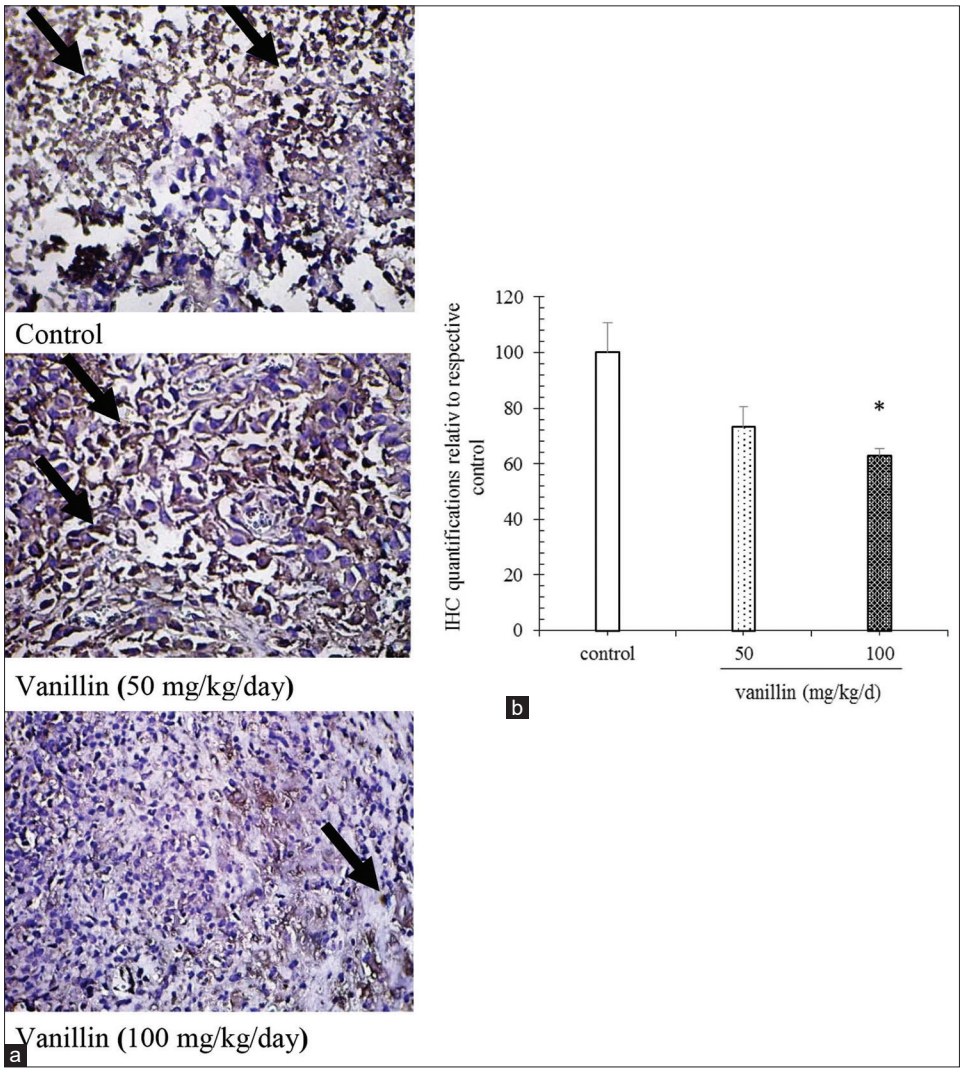

Figure 4: Immunohistochemical (IHC) evaluation of nuclear factor-κB. (a) IHC staining of different doses of vanillin, (b) IHC quantifications relative to respective treatment control in melanoma tumors. *$P < 0.05$ and Values are expressed as mean ± standard error of the mean ($n = 6–8$)

NF-κB expression and translocation of p65.[31] Vanillin by inhibiting NF-κB could reduce the expression of MMP-9, which is one of the factors required for cancer cell metastasis. NF-κB could increase the expression of MMP-9, and vanillin inhibited MMP-9 in liver cancer cells (HepG2) by reducing NF-κB.[32] The inhibitory effect of vanillin and nine other aromatic aldehydes on doxorubicin NF-κB activation was evaluated in melanoma. The *in vitro* study showed that some of these aromatic aldehydes could reduce the expression of NF-κB in A375 cells. Finally, only two compounds (not include vanillin) that had a better effect on suppression of A375 NF-κB activity were selected to treat the mice melanoma tumor (13); while in this study, we studied the effect of vanillin on reducing NF-κB expression in mouse tissue. The treatment of C57BL/6 mice with vanillin showed that vanillin could reduce NF-κB expression.

Conclusion

The present study provided evidence that vanillin could inhibit melanoma cell viability *in vitro* and reduce melanoma tumor size through inhibiting NF-κB signaling in an animal model. We observed that Vanillin in both doses (50 and 100 mg/kg) reduced melanoma tumor size compared to the controls. The results showed a decrease in NF-κB expression in vanillin groups, but only at the high dose was significantly different compared to the control group. Due to the effectiveness of vanillin in reducing tumor size and its low side effects, probably it can be used as a new drug for melanoma treatment. However, more studies are needed to find an effective dose of vanillin for melanoma treatment.

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Conflicts of interest

There are no conflicts of interest.

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