Original Article

Low-Dose Atorvastatin has Promoting Effect on Melanoma Tumor Growth and Angiogenesis in Mouse Model

Golnaz Vaseghi[1,2], Elmira Ahmadzadeh[2], Hajar Naji Esfahani[2], Majed Bahri Najafi[2], Nazgol Esmailian[2], Shaghayegh Haghjooy Javanmard[2]

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

Abstract

Background: Preclinical evidence indicates that statins possess diverse antineoplastic effects in different types of tumors. However, clinical studies have yielded conflicting results regarding the potential of statins to either increase or decrease the risk of cancer. Our objective was to examine the relationship between the dose of a treatment and its impact on melanoma tumor growth and angiogenesis in an *in vivo* setting.

Materials and Methods: Melanoma cells were injected into C57BL6 mice in four groups. They received 0, 1, 5, and 10 mg/kg of atorvastatin daily. Three others received the mentioned doses one week before the inoculation of melanoma animals. At the end of the third week, the animals were euthanized in a humane manner, and both blood samples and tumor specimens were collected for subsequent analysis.

Results: The tumor size was 1.16 ± 0.25 cm^3 in a group treated with therapeutic dose of atorvastatin and was significantly larger than that in the control group (0.42 ± 0.08 cm^3). However, there were no significant differences between the two other doses and the control group (0.72 ± 0.22, 0.46 ± 0.08 cm^3 in atorvastatin-treated groups with 5 and 10 mg/kg). The vascular density of the tumors was significantly increased in the lowest dose of the atorvastatin treatment group, similar to the results of tumor size ($P < 0.05$).

Conclusion: Atorvastatin, at low therapeutic concentrations, has been observed to stimulate tumor growth and exhibit pro-angiogenic effects. Therefore, it is advised to exercise caution and recommend clinically relevant doses of statins to patients with cancer.

Keywords: Angiogenesis, Atorvastatin, melanoma tumor

Address for correspondence: Dr. Shaghayegh Haghjooy Javanmard, Applied Physiology Research Center, Cardiovascular Research Institute, Isfahan University of Medical Sciences, Isfahan, Iran.
E-mail: shaghayegh.haghjoo@gmail.com
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Introduction

Statins belong to a group of medications that hinder the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, an enzyme crucial for cholesterol production. By doing so, they diminish the synthesis of cholesterol.[1] In addition to their cholesterol-lowering capabilities, statins have been discovered to possess pleiotropic effects on various cellular processes, including apoptosis, angiogenesis, inflammation, senescence, and oxidative stress. These effects have been observed in different types of cells, with a specific focus on cancer cells and endothelial cells.[1,2] Notably, statins play a significant role in regulating essential signaling proteins such as Rho, Ras, and Rac, thereby impacting cancer cell proliferation, migration, and survival.[3,4]

Numerous studies have presented convincing findings that indicate a significant link between the usage of statins and cancer. Preliminary research indicates that statins might enhance cancer outcomes and prolong survival time.[5] These effects have been thoroughly investigated through both laboratory experiments *(in vitro)* and studies involving live organisms (*in vivo*), encompassing a wide range of cancer

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types with diverse genetic and molecular attributes. Among these cancers are breast, prostate, colorectal, ovarian, and lung cancer.[6,7] Specifically for melanoma, research has shown that statins may have the potential for treatment, as they have been found that statins have antiproliferative, proapoptotic, and immunomodulatory effects on melanoma cell lines and mouse models.[3,8,9] Also, there are studies that show statin can enhance the therapeutic efficacy of anticancer drugs in melanoma, indicating its promising role in combination therapy.[10,11] Nevertheless, previous studies have yielded inconsistent findings. While some large-scale cardiovascular trials have indicated a reduction in melanoma incidence associated with statin use, this effect has not been consistently observed in other studies or meta-analyses examining different types of cancer.[12-15] A recent Mendelian randomization analysis revealed a reduced overall risk of cancer in individuals with variants in the HMGCR region, which are considered proxies for statin use. However, statistical significance was not attained for any specific types of cancer in the analysis.[16] Another study found that patients with melanoma taking statins had better 5-year overall survival compared to those not taking statins.[17]

Furthermore, utilizing computational drug screening via the Connectivity Map, statins were identified as a prospective category of drugs for the prevention of melanoma metastasis.[18] Several studies revealed the antiangiogenic activity of statins in tumor models. The impact of statins on angiogenesis is still uncertain, as they have demonstrated the ability to enhance the movement of mature endothelial cells and endothelial progenitor cells at lower concentrations, while displaying antiangiogenic properties primarily at higher concentrations. These inconsistencies could be attributed to the conflicting effects of atorvastatin on angiogenesis or variations in experimental conditions.[18,19] Moreover, there is a suggestion that the pro-angiogenic impact of the drug, which might contribute to skin tumor development, is unrelated to its inhibition of HMG-CoA reductase and could be directly mediated by atorvastatin itself.[19]

According to these discordances in the statin effect, a new review article explains that perhaps the main reason for the conflicting results in studies on statins' impact on cells is the varying doses used in different experiments. When lower statins are used, they have been found to have anti-senescence and antiapoptotic effects. However, at higher doses, statins can actually have the opposite effect. Many studies on cancer cells have used high doses of statins, which have been shown to have cytotoxic and cytostatic effects. Therefore, it is important to consider the dose of statins used in future studies to assess their effects on cells[3] accurately. In this study, we aimed to investigate the effect of atorvastatin, one of the most potent and commonly prescribed lipophilic statins, with doses near the therapeutic doses, on the growth and angiogenesis of mouse melanoma. We also tried to understand whether atorvastatin has any preventive effects on melanoma.

Materials and Methods

Cell culture

B16-F10 mouse melanoma cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine, 100 U/ml penicillin, and 10% fetal bovine serum. The cells were maintained at 37°C in a 5% CO2 atmosphere. Once the melanoma cell monolayer reached approximately 80% confluence, it was washed and detached using a solution of phosphate-buffered saline (PBS) containing 0.25% trypsin and 0.03% ethylenediaminetetraacetic acid (EDTA). Subsequently, the cells were briefly centrifuged at 100 g to form a pellet. After discarding the supernatant, the cell pellets were resuspended in PBS, and the cell count was determined.

In vivo experiments

The animals involved in this study were treated according to the guidelines specified in the "Guide for the Care and Use of Laboratory Animals" (8th edition, National Academies Press), ensuring their proper care and ethical handling.

Animal experiments were conducted on C57BL6 mice, with weights ranging from 20 to 25 grams. These mice were housed in a controlled environment at a temperature of 22-24°C, following a regular light/dark cycle. Throughout the experiments, the mice had free access to food and water. The study comprised seven randomly formed groups, each consisting of eight mice. Ethical guidelines specified by the Institutional Ethics Committee of Isfahan University of Medical Sciences were strictly followed in the treatment of all the mice involved in the research. To induce melanoma, syngeneic B16-F10 melanoma cells were subcutaneously injected into the flank of all C57BL6 mice at a concentration of 106 cells in 200 µl of PBS. Care was taken to ensure that an equal amount of cells was injected into each animal, minimizing any variations in tumor development resulting from differences in cell inoculation. Drug-treated mice were administered with atorvastatin (Dr. Abidi Company, Iran) dissolved in 0.1% dimethyl sulfoxide (DMSO)/PBS solution (w/v), while control animals were administered intraperitoneal (IP) with a vehicle. To ensure consistent bioavailability and eliminate variations, IP administration was selected. This route allows for complete absorption of the administered substance. In the treatment groups, the administration of atorvastatin commenced simultaneously with the injection of B16-F10 cells and continued for a duration of 21 days. The study involved 48 mice divided into seven groups. The first group was a control group that received daily 200 µl normal saline IP. The second, third, and fourth groups, respectively, received 1, 5, and 10 mg/kg of atorvastatin daily IP starting seven days before cancer induction. The fifth, sixth, and seventh groups, respectively, received 1, 5, and 10 mg/kg doses of atorvastatin daily starting on the day of cancer induction until the last day. All animals were euthanized by pentobarbital overdose on the 21st day. Following euthanasia, the tumors were carefully dissected, and their volumes were determined using the formula V = (4/3 × π × (a)2 × (b), where "a" represents half

of the minor axis and "b" denotes half of the major axis of the prolate spheroid. In the prevention groups, atorvastatin was administered at doses of 1 mg/kg, 5 mg/kg, and 10 mg/kg for a period of one week before the induction of melanoma cells.[20]

Capillary density assessment

Tumor samples were initially fixed in 10% neutral-buffered formalin for an overnight period to perform immunostaining on endothelial cells. The materials were then cut into slices with a thickness of 5 m and fixed in paraffin. These sections were then deparaffinized using xylene and gradually rehydrated through a series of alcohol solutions. To block endogenous peroxidase activity, a solution of 3% H2O2 in methanol was applied. To retrieve antigens, microwave irradiation was employed for a duration of 15 minutes in a 10 mM citrate buffer with a pH of 6.0. Following a cooling period of 20 minutes, the sections were rinsed with PBS. To minimize nonspecific staining, a protein block (RE7120) was utilized. Following that, the sections were exposed to monoclonal antibodies targeting mouse CD31 (Novocastra) at a dilution of 1/100 for 60 minutes. A horseradish peroxidase enzyme-labeled polymer connected to a secondary antibody specific to mice was used to identify the attached antibody. Diaminobenzidine was used as the chromogen to visualize the stained areas. Hematoxylin was utilized for counterstaining. Positive controls consisted of paraffin-embedded sections from normal samples, while negative controls were treated with PBS instead of the primary antibody. Capillary density was evaluated at a magnification of ×400 in five different fields from each tissue sample. The number of vessels observed in those five fields was averaged and reported as the resulting vessel count.

Reverse transcriptase–polymerase chain reaction (RT-PCR)

To evaluate the expression of the vascular endothelial growth factor (VEGF) gene, total RNA was isolated from melanoma samples of mice using the Rneasy Mini Plus Kit (Qiagen, Valencia, CA, USA), following the provided instructions. Spectrophotometer measurements and gel electrophoresis were used to evaluate the RNA's purity. Then, using oligo-dT primers and RevertAid™ Reverse Transcriptase (Fermentas, Vilnius, Lithuania), cDNA was created. As internal controls for quantitative real-time RT-PCR, unique primers for tau and stathmin mRNAs were employed. The Rotor-Gene 6000 (Qiagen, Hilden, Germany) was used for the amplification procedure, and the Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, Vilnius, Lithuania) was used. The PCR cycling conditions were 45 cycles of amplification that involved denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. The first denaturation phase took place at 95°C for 10 minutes. By examining PCR results on a 1.5% agarose gel stained with ethidium bromide and viewed under ultraviolet light, their identities were verified. Primers for nuclear factor kappa B (NF-kB) were designed using Beacon Designer, and their sequences are provided below.

NF-kB forward primer: (5′-ACACGAGGCTACAACTCTGC-3′)

Reverse primer: (5′- GGTACCCCCAGAGACCTCAT-3′)

GAPDH forward primer: (5'-TGGAGAAACCTGCCAAGTATGATG-3')

Reverse primer: (5'-AGTGGGAGTTGCTGTTGAAGTC-3')

Blood cholesterol concentration

Blood plasma was isolated from the samples, and the cholesterol level was measured by a kit (over-the-counter treatment) according to its protocol with a NanoDrop device and a calorimetric system. For this purpose, the blood tubes were centrifuged for 10 minutes, and the serum was isolated. Ten microliters of plasma was deposited in the microtip, and 1 ml of the enzyme was added. A microtip was considered standard (S), containing 10 µl of the standard cholesterol and 1 ml of enzyme reagents. In another microtip with B, only 1 ml of the enzyme was deposited. The contents of the tubes were mixed and placed them for 10 minutes at 37°C. Then, the intensity of the paint was read in tubes containing plasma and standard in front of tube B at a wavelength of 520 nm. Using the following formula, the cholesterol concentration was measured.

Cholesterol level (mg/dl) = × standard cholesterol concentration (mg/dl)

Statistical analysis

The Kolmogorov–Smirnov test was used to determine whether the data had a normal distribution and were reported as mean ± standard deviation. One-way analysis of variance (ANOVA) was used to compare groups, and the Bonferroni procedure was used as a *post hoc* analysis. A *P*- value of less than 0.05 was regarded as statistically significant. The SPSS 16 program (SPSS Inc.) was used to conduct all statistical analyses.

Results

Tumor volumes

As shown in Figure 1A, tumor volumes increased in the therapeutic dose of atorvastatin (1 mg/kg) group with a mean of 1.16 ± 0.25 compared to the control group with a mean of 0.48 ± 0.08. In the prevention group, no significant differences were seen between groups [Figure 1].

Capillary density

The results of capillary density showed that angiogenesis increased in the treatment group with the dose of 1 mg/kg atorvastatin (7.3 ± 0.93). However, no significant differences were seen between other and prevention groups [Figure 2].

Expression of NF-kB

Figure 3 shows that the expression of NF-kB in all three doses of 1, 5, and 10 mg/kg was reduced relative to the control, and the lowest dose of 1 mg/kg was lower than other doses [Figure 3].

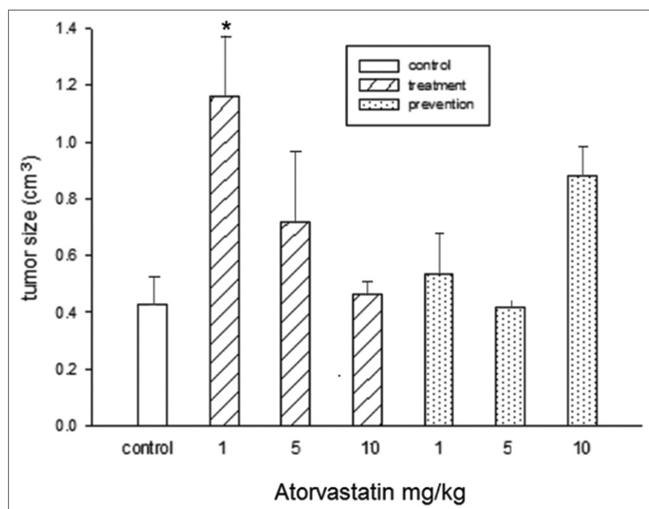

Figure 1: Effect of different doses of atorvastatin on tumor size, in prevention and treatment groups. The results of the tumor volume shown in the diagram indicate that the melanoma tumor volume has increased in the treatment group with a decrease in the drug dose; thus, increase in the treatment group with a dose of 1 mg of atorvastatin with an average of 1.16 ± 0.25 has a significant difference compared to the control group with an average of 0.42 ± 0.08. In the prevention group, the mass volume in none of the doses was significantly different from the control. Data have been shown as mean ± SEM. *$P < 0.05$ in comparison with the control

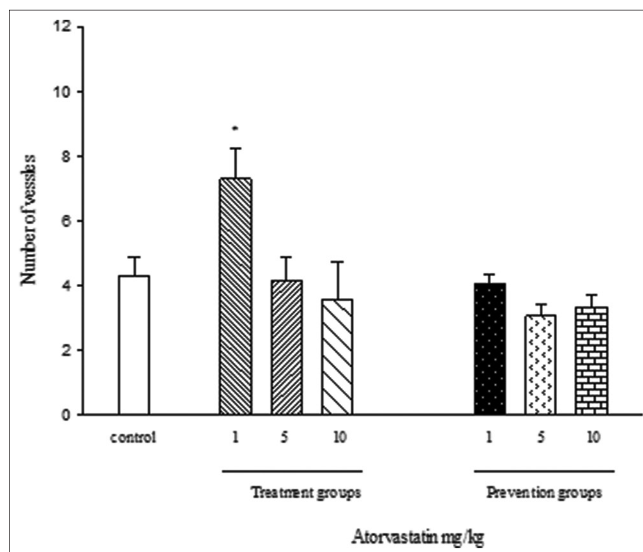

Figure 2: Effect of atorvastatin on tumor angiogenesis. Number of vessels produced with different doses of atorvastatin, in prevention and treatment groups. The results of examining the amount of angiogenesis of masses are shown in Figures 3 and 4. Data analysis was performed by one-way ANOVA method and showed the amount of angiogenesis in treated masses receiving 1 mg of atorvastatin with an average of 3.7 ± 0.93 compared to all groups except the treated group receiving 5 mg. It has a significant difference with the mean of 13.4 ± 0.73, and it has increased the angiogenesis of melanoma tumor. Data have been shown as mean ± SEM. *$P < 0.05$ in comparison with the control

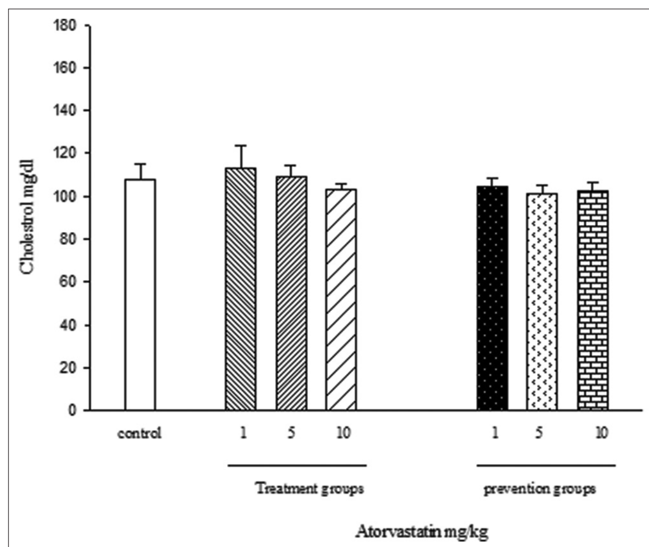

Figure 3: Cholesterol concentration in mouse blood with different doses of atorvastatin in prevention and treatment groups. According to the statistical analysis, there is no significant difference between the atorvastatin and control groups. Data have been shown as mean ± SEM

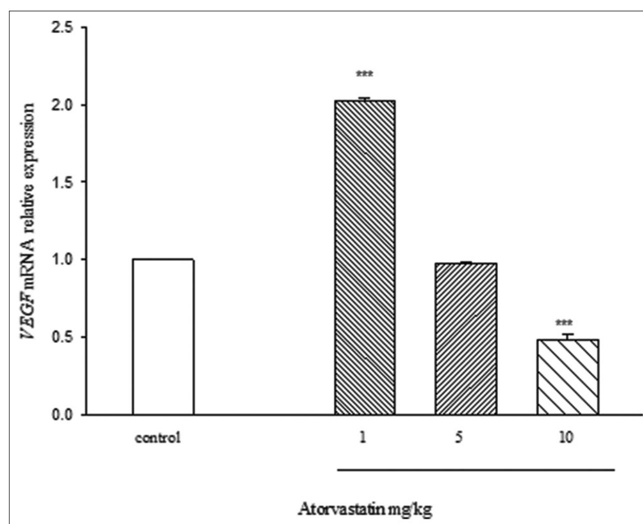

Figure 4: Expression of VEGF mRNA in atorvastatin treatment groups. Data have been shown as mean ± SEM. ***$P < 0.001$ in comparison with the control

Plasma cholesterol

There were no significant differences between the plasma concentration of cholesterol in different doses of atorvastatin and also the control group [Figure 4].

CD31 Expression

Figure 5 displays immunohistochemistry results for CD31 staining in various treatment groups with atorvastatin at different dosages. These groups include the control, treatment (at 1 mg/kg, 5 mg/kg, and 10 mg/kg), and prevention (at 1 mg/kg, 5 mg/kg, and 10 mg/kg). This figure provides insights into atorvastatin's impact on CD31 expression, a marker linked to vascular health and angiogenesis. Differences in staining patterns among the groups will be discussed, offering insights into atorvastatin's potential benefits in regulating vascular function and angiogenesis [Figure 5].

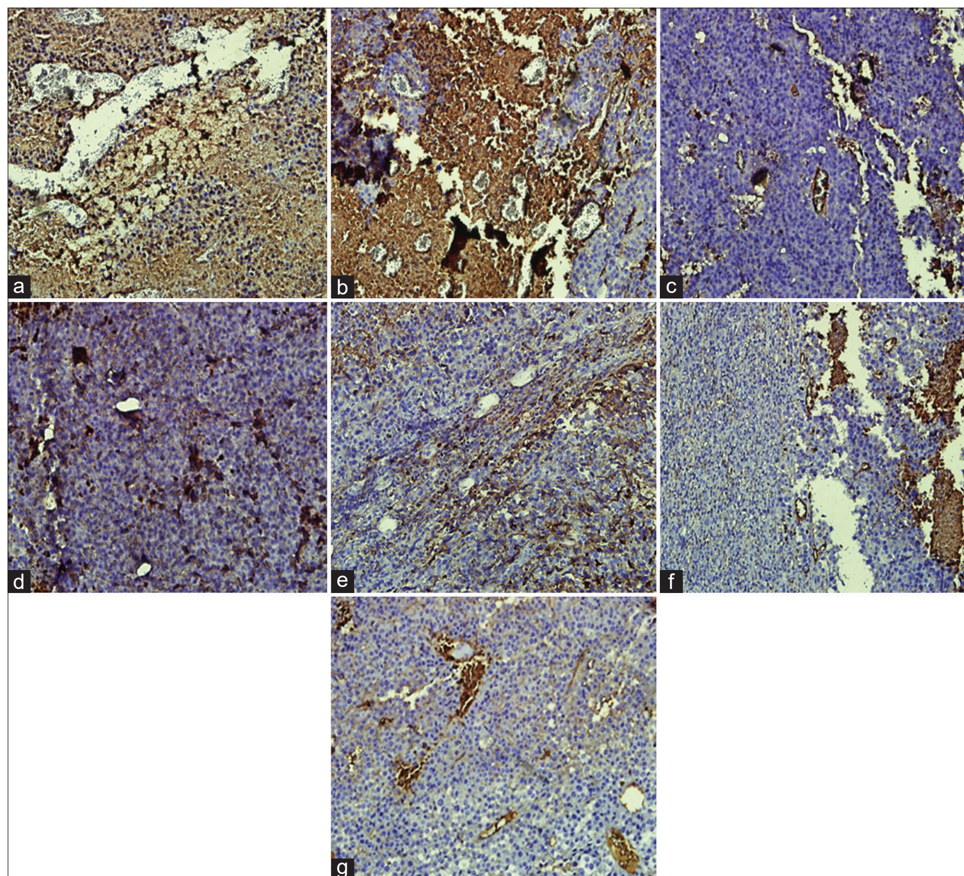

Figure 5: Immunohistochemistry of CD31 in different treatment groups. (a) Control group (b-d) treatment group (1, 5, and 10 mg/kg of atorvastatin), respectively, (e-g) prevention group (1, 5, and 10 mg/kg of atorvastatin)

Discussion

Our study aimed to investigate the effects of statins on tumor growth and angiogenesis *in vitro*. Surprisingly, we found that atorvastatin, a commonly prescribed statin, can actually promote these processes at the lowest dose tested. We showed that statin can promote tumor growth and angiogenesis in the lowest dose of the atorvastatin treatment group (1 mg/kg), equivalent to 40-80 mg/kg, which has been used in routine clinical practice for patients with hypercholesterolemia. Our findings are supported by previous studies that demonstrated statin's antiapoptotic and anti-senescence effects. Various research studies have confirmed the impact of statins on the progression of the cell cycle, senescence, and apoptosis in endothelial cells.[1] For example, it was shown that proliferation, migration, and tube formation of endothelial colony-forming cells (ECFCs) were enhanced by pravastatin.[21] In an epidemiology study, statin use did not affect overall survival, but it may impact the survival of patients with melanoma based on gender. The timing, duration, and dosage of statin use did not significantly affect the risk of death. However, male statin users may have better survival rates compared to non-users.[13]

Furthermore, according to our findings in different statin concentrations, there are findings suggest that the concentration of statins used can have varying effects on cellular processes. Numerous studies have examined how statins affect Bcl-2, finding that high doses of the medication lower Bcl-2 levels and trigger apoptosis. However, lower concentrations of statins have been found to increase Bcl-2 expression and suppress cell death.[22] In breast cancer and melanoma tumors, treatment with pitavastatin at a concentration of 10 μM has demonstrated the ability to enhance the radiation's effects on cellular senescence. Conversely, the effects of pravastatin were restored when supplemented with 5 mM mevalonic acid.[24] Likewise, in prostate epithelial cells, the application of 100 nM concentration of simvastatin resulted in cytostatic and senescent effects, while also partially inducing apoptosis. However, at a higher concentration of 10 μM, simvastatin exhibited cytotoxic effects on both normal and cancer cells. Notably, the combination of low-density lipoprotein (LDL) cholesterol and mevalonate supplementation effectively rescued the cytostatic and cytotoxic effects caused by 10 μM simvastatin.[23] These findings suggest that the concentration of statins used can have varying effects on cellular processes and should be considered when prescribing the drug.

In contrast to our findings, cytotoxic (apoptotic) and cytostatic effects of statins in cancer cells have been confirmed in various studies.[2,24,25] It was shown that atorvastatin use at the dosage of 1.16 μM to 4.3 μM induced apoptosis in

MDA-MB-231 cells.[26] Another study shows the cytotoxic activity of simvastatin in T47D breast cancer cell lines and its effect on cyclin D1.[27] A comprehensive umbrella review was performed, examining previous meta-analyses to assess the correlations between statin use and cancer incidence. After re-analyzing data from 43 meta-analyses, it was discovered that among the 18 cancer incidence associations examined, 10 exhibited a statistically significant preventive effect linked to the usage of statins.[28]

Also, atorvastatin in all the doses examined in this study could not cause a significant change in serum cholesterol. We also demonstrated that statins could increase the expression of NF-kB and the VEGF gene in melanoma tumors in these therapeutic doses in animal models. The pro-angiogenic effect of low-dose atorvastatin can be due to its effect on the amount of nitric oxide (NO) synthesis[29] and the proliferation and migration of endothelial and endothelial progenitor cells. Endothelial nitric oxide synthase (eNOs) or NO affects cell cycle apoptosis and cancer progression.[30,31] However, some of its effects depend on the concentration. In high concentrations, it increases the phosphorylation of P53, which has an inhibitory effect on the growth of endothelial cells and also induces apoptosis. It also plays a role in inhibiting NF-kB and inhibits cancer cell resistance and metastasis.

However, in low concentrations, it is pro-angiogenic and thus supports the growth and metastasis of cancer tumors by increasing VEGF. Of course, different types of tumors have shown different sensitivity to NO.[32] Similar to this, another study found that atorvastatin at five and ten micrograms per milliliter greatly boosted the expression of the VEGF-A gene in the HN13 cell line. According to this study, atorvastatin causes angiogenesis and oxidative stress to increase in oral squamous cell carcinomas.[33] Another study found that atorvastatin has a strong antiangiogenic effect and induces apoptosis in glioma spheroids. At a concentration of 10 μM, it downregulated the expression of VEGF, CD31, and Bcl-2, while increasing the expression of caspase-3.[34] Other studies have also suggested that the antiangiogenic effect of statins may be linked to the induction of endothelial cell apoptosis.[35] This difference in the results can be due to the difference in the type of cancerous tissue studied and the difference in their sensitivity to statin; also, the difference in the injection method and the duration of the study can have an effect.

Conclusion

However, our study adds a new layer of complexity to the relationship between statin use and cancer. While some studies have shown a preventive effect of statins on cancer incidence, our findings suggest that the impact of statins on tumor growth and angiogenesis may be context-dependent. It is important to note that our study was conducted *in vivo* in a mouse model. Additionally, our study only evaluated the effects of atorvastatin, and other statins may have different effects on cell behavior. Despite these limitations, our study highlights the need for a more nuanced understanding of statins' effects on cancer, specifically melanoma cancer. Rather than assuming that all statins have a uniform effect on cell behavior, it is important to consider the specific cellular and molecular context in which they are used. This information could ultimately lead to more targeted and effective use of statins in treating cancer and other diseases. So, clinically relevant doses of statins should be recommended with more caution to patients with cancer.

Statement of ethics

The Isfahan University of Medical Sciences ethics committee has accepted the study, which was conducted in accordance with the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines.

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Conflicts of interest

There are no conflicts of interest.

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