Original Research

Neferine induces mitochondrial dysfunction to exert anti-proliferative and anti-invasive activities on retinoblastoma

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Impact statement

Retinoblastoma (RB) is the most common primary intraocular malignancy of infants and childhood. The difficulty of RB treatment lies in its strong resistance to chemotherapy and delayed diagnosis caused by imperceptible pathological changes. This study evaluates the mitigation role of Neferine on RB *in vitro* and *in vivo*. The result is that high-dose Neferine could decrease retinoblastoma angiogenesis in association with a significant inhibition on tumor growth and invasion. These findings suggested that Neferine could be a new treatment or adjuvant against RB.

Abstract

Retinoblastoma is common primary intraocular malignancy of infants and childhood. Neferine is a major bisbenzylisoquinoline alkaloid derived from the lotus plumule in *Nelumbo nucifera*. This study evaluated the mitigation role of Neferine on retinoblastoma *in vitro* and *in vivo*. Xenotransplantation model was established by injecting WERI-Rb-1 cells subcutaneously. Upon induction of retinoblastoma , mice were intraperitoneally injected with Neferine (0, 0.5, 1, 2 mg/kg) or ethanol every 3 days for 30 days. Tumor weight and tumor volume were measured every three days and compared between four groups. Then, mice were sacrificed and immunohistochemical examination was performed to compare Ki67, VEGF content between groups. WERI-Rb-1 cells were used for *in vitro* experiments and the anti-angiogenic role of Neferine was assessed by analyzing nodes/HPF number. In WERI-Rb-1 xenotransplantation model, compared with control group, 1 mg/kg Neferine treatment significantly inhibited tumor weight (0.39 ± 0.04 g vs. 0.25 ± 0.03 g, $P < 0.05$) and tumor volume (2163 ± 165 mm^3 vs. 1276 ± 108 mm^3, $P < 0.05$) after 30 days. Compared with ethanol-injected mice, 2 μM Neferine treatment significantly enhanced apoptosis rate ($2.1 \pm 0.6\%$ vs. $14.6 \pm 2.6\%$, $P < 0.05$), accompany downregulation of Ki67 ($0.09 \pm 0.02\%$ vs. $0.01 \pm 0.004\%$, $P < 0.05$) and VEGF ($0.28 \pm 0.04\%$ vs. $0.05 \pm 0.03\%$, $P < 0.05$) expression. Additionally, 2 μM Neferine treatment significantly decreased JC-1 red/green percentage. High-dose Neferine could decrease retinoblastoma angiogenesis in association with a significant inhibition on tumor growth and invasion. These findings suggested that Neferine could be a new treatment or adjuvant against retinoblastoma.

Keywords: Retinoblastoma, mitochondrial dysfunction, Neferine, JC-1 red/green, apoptosis, anti-angiogenic

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Introduction

Retinoblastoma (RB), which represents 3% of all childhood cancers, is the most common primary intraocular malignancy of infants and childhood. The characteristic symptoms of RB are strabismus or leukocoria. In late stage of the disease, the child may exhibit buphthalmos, proptosis, or hypopyon. In developing countries, the mortality from RB is about 70%.[1,2] Retinoblastoma tumor formation usually starts with mutation in both alleles of the retinoblastoma tumor suppressor gene RB1, followed by a spectrum of other genetic alterations correlated with the clinical stage and pathologic findings of the tumor. To date, the difficulty of RB treatment lies in its strong resistance to chemotherapy and delayed diagnosis caused by imperceptible pathological changes. Hence, further understanding the molecular mechanism(s) of RB pathogenesis and discovery of new drugs are urgently needed to improve the clinical therapeutic effects.

Mitochondria are important dynamic organelles that participate in various activities such as transport, fusion, and fission, and play a key role in oxidative phosphorylation and energy metabolism. The production of reactive oxygen species, the production of ATP, and the buffering of cytosolic calcium are the three main functions of mitochondria. Mitochondrial dysfunction is considered as the

cause of systemic heterogeneity, phenotypic abnormalities, and genetic variation.[3,4] Risk factor for graves ophthalmopathy (GO) patients may have adverse effects on the mitochondrial biogenesis, such as cigarette smoking.[5]

Neferine (Nef) is a major bisbenzylisoquinoline alkaloid derived from the lotus plumule (embryos of the seeds) in *Nelumbo nucifera*. Evidences have shown that Nef individuals possess extensive pharmacological properties in many diseases, such as anti-cancer, anti-oxidant, anti-inflammatory, anti-multidrug resistance, and neuroprotective effects.[6–8] Kalai *et al.*[9] found that Nef enhances the anti-tumor effect of cisplatin via ROS- mediated non-canonical autophagy and mitochondria-mediated apoptosis pathway in lung cancer cells. Wu *et al.*[10] reported that Nef might exert its therapeutic potential for ischemic stroke through mitochondrial protection and the underlying mechanism may be critically implicated in Nrf2 signaling.

However, whether Nef played anti-cancer role on retinoblastoma remains uncertain. Therefore, the purpose of this study was to evaluate the therapeutic effect of Nef on retinoblastoma *in vitro* and *in vivo*. In this study, WERI-Rb-1 cells were selected as experimental cell line, and explored the anti-tumor effect of Nef on WERI-Rb-1 cells, as well as uncovered whether Nef induced mitochondrial dysfunction and further caused apoptosis. The data demonstrated that high-dose Nef could decrease retinoblastoma angiogenesis in association with a significant inhibition on cell proliferation, invasion, migration, and induced apoptosis in WERI-Rb-1 cells. Notably, we found that Nef exhibited the anti-tumor effect on WERI-Rb-1 cells by inducing mitochondrial dysfunction. Xenotransplantation model was established by injecting WERI-Rb-1 cells subcutaneously. The results demonstrated that Nef treatment significantly inhibited tumor weight and tumor volume. These findings suggested that Nef could be a new treatment or adjuvant against RB.

Materials and methods

Materials

Nef (purity > 98%), purchased from Best Biotechnology Co., Ltd (Chengdu, China), was dissolved in 0.1% ethanol.[11] The stock and working concentrations of Neferine used in this study were respectively 800 μM and 200 μM. Matrigel Matrix Basement Membrane HC was purchased from Binzhi Biological Technology Co., Ltd (Shanghai, China). JC-1 kit was purchased from Solarbio life sciences Technology Co., Ltd (Beijing, China). DAPI staining solution (C1005) was purchased from Beyotime Biological Technology Co., Ltd (Shanghai, China). RPMI1640 medium and fetal bovine serum (FBS) were purchased from GenePharma Co., Ltd (Shanghai, China).

Cell culture

WERI-Rb-1 (https://web.expasy.org/cellosaurus/CVCL_1792) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and were cultured in RPMI1640 medium supplemented with 10% FBS in an atmosphere containing 5% CO_2 at 37°C. Cells of the third to fifth passage were used in experiment.

Animals

The animal experiments in present study were carried out according to the Guidelines for the Care and Use of NIH Experimental Animals and approved by the First Affiliated Hospital of Zhengzhou University. Twenty female athymic nude mice (Four-week-old) were provided by the Animal Center of the First Affiliated Hospital of Zhengzhou University. All rats were kept in the departmental animal house on a 12-h light/dark cycle under a controlled temperature (24 ± 0.5°C) with free access to food and water in the whole process.

Xenotransplantation model

Twenty female athymic nude mice (four-week-old) were used for the xenotransplantation model. Mice were injected subcutaneously in the right subaxillary region with 1×10^6 (0.3 mL) WERI-Rb-1 cells mixtured with Matrigel Matrix HC[12] and RPMI with 20% bovine serum (Invitrogen). One week after subcutaneous WERI-Rb-1 cell injection, mice were assessed for successfully transplantation of tumors. Mice transplanted successfully ($v > 200\ mm^3$) were divided into four groups and injected intraperitoneally with Nef (0, 0.5, 1, 2 mg/kg) or an equal volume of ethanol every 3 days for 30 days.[13] Uninformed operators measured tumor weight, tumor volume,[14,15] and body weight every three days. Thirty days after xenotransplantation, tumors were harvested to measure tumor weight and performed immunohistochemical assessments.

Cell viability assay

Viability of WERI-Rb-1 cells were examined by the Cell-Counting Kit-8 (CCK-8) (Kumamoto, Japan). Upon the action of the electron carrier (1-Methoxy PMS), WST-8 contained in the CCK-8 reagent is reduced to a highly water-soluble yellow formazan dye by the dehydrogenase in the mitochondria of cell and the amount of formazan produced is proportional to the number of living cells. Concretely, 200 μL WERI-Rb-1 cells medium were seeded at a density of 5×10^3 cells/well into 96-well plates (Corning, NY, USA). Subsequently, the cells were treated with different concentrations of Nef (0, 0.1, 0.2, 0.5, 1, 2, 4, 8, 20, 50, 100, 200 μM) for 24 h in an incubator at 37°C with 5% CO_2. Next, 10 μL/well of CCK-8 dye (5 mg/mL) was added, and then the plate was incubated at 37°C for an additional 4 h in a 5% CO_2 atmosphere. Each group consisted of five parallel wells. Then the absorbance was measured by microplate reader (Genios TECAN, Männedorf, Schweiz) 450 nm and the observation time lasts one week at the same time every day.

Cell proliferation assay

The proliferation of WERI-Rb-1 cells was measured using a bromodeoxyuridine (BrdU) kit (Thermo Fisher, USA). The cell proliferation cycle includes G1, S, G2, and M stages. The S stage is mainly responsible for the synthesis of genomic DNA. The analysis of DNA synthesis can directly reflect the

S-phase progress of cells. BrdU is a thymine analog, which can be embedded in the newly synthesized DNA sequence in DNA synthesis. The anti-BrdU specific antibody can be used to analyze the situation of newly synthesized DNA. Briefly, Nef-treated WERI-Rb-1 cells were seeded into 96-well plates (5×10^3 cells/well) and incubated overnight at 37°C. The cells were then marked with a 1 mg/mL BrdU solution for 3 h. In addition, cells were denatured using FixDenta solution for 30 min, and then incubated with peroxidase-conjugated anti-BrdU antibodies for 90 min.[16] Nuclei were counterstained with DAPI (Beyotime, Shanghai, China). The number of BrdU positive cells was observed under a fluorescence microscope (Olympus, Japan). Under blind magnification (×400), BrdU-positive cells were counted as five fields per section.

JC-1 staining

JC-1 is one kind of dye that targets to mitochondrion is used as a reporter for membrane potential. JC-1 binds to mitochondria and shows a change in fluorescence that is a function of the membrane potential that drives the aggregation of the dye. The ratio measurement of the two fluorescence intensities is then used as a measure of the relative membrane potential. Both dyes are well-suited for real-time imaging and report changes in membrane potential based on experimental procedures, further reflecting the state of mitochondrial function.[4] JC-1 staining of WERI-Rb-1 cells was referred to previous paper.[17] DAPI (Beyotime, Shanghai, China) was added to make the final concentration at 10 mg/L to visualize the nuclei. They were assessed using 488 nm excitation and also used for confocal microscopy (Olympus, Japan). Samples were assessed by fluorescence registration (red and green) and NIS-Elements software.

Detection of oxidative stress index

Cells and tumor tissue samples were collected to examine oxidative stress index. The contents of SOD, MDA, and GSH were measured using kit and measured strictly according to the kit instructions (Qiaoyu Biotechnology Co., Ltd, Shanghai, China).

Transwell assay

Transwell assay was performed to examine the invasive capacity of WERI-Rb-1 cells. The matrigel was applied to the middle bottom of the upper chamber and air-dry at room temperature. Upon incubating for 24 h with serum-free medium, cell suspension (200 mL/chamber) was added into the upper chambers. The lower chambers were filled with 600 μL medium containing 10% FBS. As incubation at 37°C for 24 h, the chambers were removed and washed twice with PBS. The residual cells were cleared. After being fixed with 95% alcohol and stained with crystal violet, cells were examined by microscope (Leica, Germany) and the average amount of invasive cells was recorded. The experiments were independently repeated in triplicate.

Microtube formation assay

WERI-Rb-1 cells were retreated with Nef at four concentrations (0, 1, 2, and 4 μM) for 48 h. Matrigel was mixed with serum-free RPMI medium pre-cooled at 4°C in a ratio of 1:1. The mixture was laid on the bottom of 24-well plate, 300 μL per hole, and cured in 5% CO_2 and 37°C incubator for 30 min. The WERI-Rb-1 cells treated with Nef were inoculated into the above-mentioned gelatinized 24-well plates after adjusting the concentration to 1.2×10^5/mL in serum-free RPMI medium. The microtubule structure was observed every 4 h. Five visual fields were randomly taken under microscope (100 ×). Microvision Saisam software was used to analyze the images, and the microtubule-like structures with five visual fields per hole were counted. The experiments were independently repeated in triplicate.

Flow cytometry

Upon treated with each concentration of Nef, the cell apoptosis rate in sphere-forming cells was measured referring the previous report. Briefly, the detection of cell apoptosis rate was performed with the manufacturer's protocol and measured by flow cytometry analysis.

Immunohistochemistry

The expressions of Ki67 and VEGF in xenograft nude mouse model were evaluated by the method of immunohistochemistry. Upon dewaxing, rehydration, and repair, sections were sealed with 5% normal goat serum for 1 h and co-incubation with primary antibodies Ki67 (ab15580, 1:500), VEGF (ab2350, 1:50) overnight at 4°C. Then, sections were treated according to the instructions of manufacturer and analyzed by microscopy.

Western blot analysis

Cells were collected after pretreatment with Nef. After rinsing, cells were treated with radioimmunoprecipitation assay lysis buffer dissolving protease inhibitors. Western blot was determined by Poornima *et al*.[18] The primary antibodies were as follows: Ki-67 (ab15580, 1:500), Survivin (ab469, 1:5000), VEGF (ab2350, 1:1000), Bax (ab32503, 1:1000), Bcl-2 (ab196495, 1:500), caspase-3 (ab13847, 1:500), cleaved caspase-3 (ab2302, 1:500), cleaved caspase-9 (ab2324, 1:500), caspase-9 (ab52298, 1:500), c-Myc (ab32072, 1:1000). The relative protein level was analyzed by ImageJ software. The experiments were independently repeated in triplicate.

Statistical analysis

The statistical analysis was conducted with SPSS 21.0 (SPSS, Inc., Chicago, IL, USA). All experiments were repeated at least three times. Data were presented as the mean ± SD. Multiple sets of data were analyzed by one-way ANOVA followed by Bonferroni *post hoc* test. Differences were considered statistically significant when $P < 0.05$.

Results

Selection of the optimal therapeutic dose of Nef

In this part, Nef solutions were attenuated to diverse dosages ranging from 0.1 to 200 μM and were used to process

WERI-Rb-1 cells for 24 h to select optimal treatment dose. As shown in Figure 1(b), Nef had no effect on cell viability in the dose range of 0.1–4 μM and decreased cell viability significantly in the dose range of 8–200 μM. The result indicated that Nef in this concentration range of 0.1–4 μM was not toxic to WERI-Rb-1 cells. Hence, 0, 1, 2, 4 μM Nef was used in subsequent experiments.

Nef treatment inhibited proliferation of WERI-Rb-1 cells

As shown in Figure 2(a), BrdU (+) cells (%) green was decreased as the increasing of Nef concentration. Compared with control group, 2 μM Nef treatment significantly decreased BrdU (+) cells (%) green (Figure 2(a)). Correspondingly, cell apoptosis rate (%) was increased as the increasing of Nef concentration and 2 μM Nef treatment increased cell apoptosis rate (%) significantly (Figure 2(b)). The protein expression levels of Ki67 and Survivin were detected by WB. As shown in Figure 2(c), compared with control group, 2 μM Nef treatment significantly downregulated the protein expression levels of Ki67 and Survivin. These results suggested that Nef inhibited proliferation of WERI-Rb-1 cells and promoted apoptosis rate of WERI-Rb-1 cells.

Nef treatment attenuated the invasive ability of WERI-Rb-1 cells

The invasion and microtubules formation ability of WERI-Rb-1 cells were evaluated in this part. As shown in Figure 3 (a) and (c), upon Nef treatment, the invasive ability of WERI-Rb-1 cells was reduced in a dose-dependent manner and invasive cells per field decreased obviously. As shown in Figure 3(b) and (d), compared with control group, 2 μM Nef treatment inhibited the formation of microtubule-like structures and number of nodes/HPF reduced in a dose-dependent manner. The number of microtubule-like structures in each treatment was analyzed by Microvision Saisam software. According to the result of WB assay, relative protein level of VEGF was obviously downregulated by Nef treatment in a dose-dependent manner. These results indicated that Nef (≥ 2 μM) can restrain the athletic ability of WERI-Rb-1 cells.

Nef treatment induced mitochondrial dysfunction and WERI-Rb-1 cells apoptosis

In this part, mitochondrial membrane potential was analyzed by JC-1 staining. As shown in Figure 4(a), JC-1 red/green percentage reduced following Nef treatment. 2 μM Nef treatment obviously decreased JC-1 red/green percentage compared with control group. The result suggested that Nef treatment induced abnormal changes in mitochondrial membrane potential. Compared with the control group, Nef treatment obviously suppressed the production of SOD and GSH, conversely, promoted the content of MDA (Figure 4(b)). The result illustrated that Nef treatment caused oxidative stress in WERI-Rb-1 cells. As shown in Figure 4(c), compared with control group, Nef treatment downregulated Bcl-2 and c-Myc protein expression levels and upregulated Bax protein expression level. Additionally, Nef treatment expedited the cleavage of Caspase-3 and Caspase-9. Briefly, Nef treatment increased the ratio of Bax/Bcl-2, cleav Cas3/Cas3, and cleav Cas9/Cas9. Together, these results illustrated that Nef induced mitochondrial dysfunction and WERI-Rb-1 cells apoptosis.

Nef treatment suppressed tumors formation in vivo

In this part, xenograft tumors formed by subcutaneously injected WERI-Rb-1 cells into the right thigh of nude mouse. Four concentration gradients of Nef (0, 0.5, 1, 2 mg/kg) interfered with tumor formation. As shown in Figure 5(a), Nef treatment group tumor sizes are significantly smaller than the control group. Tumor weight reduced significantly with the increasing of Nef dose (Figure 5(b)). Tumor volume was significantly smaller by Nef dose compared with the control group (Figure 5(c)). However, the body weight change of nude mice was not obvious (Figure 5(d)). As shown in Figure 5(e) and (f), the content of Ki67 and VEGF decreased dramatically in a dose-dependent manner compared with the control group. Compared with the control group, Nef treatment obviously inhibited the production of SOD and promoted the content of MDA *in vivo* (Figure 5(g) and (h)). These results suggested that Nef inhibited the oncogenesis *in vivo*.

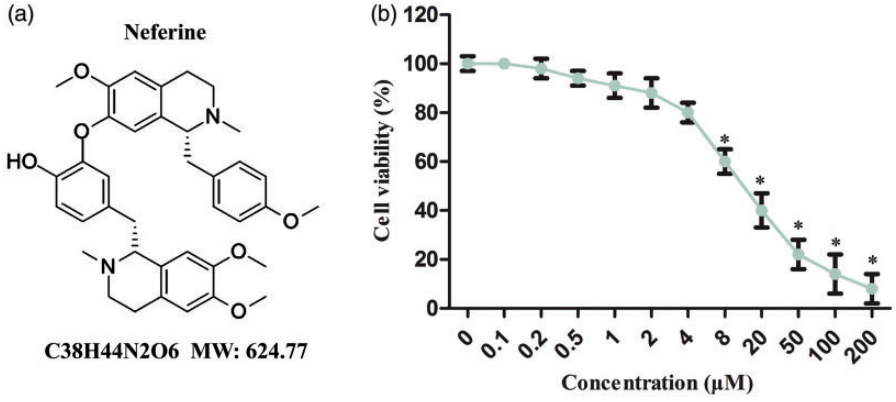

Figure 1. Selection of the optimal therapeutic dose of Nef. WERI-Rb-1 cells were treated with different concentrations of Nef (0, 0.1, 0.2, 0.5, 1, 2, 4, 8, 20, 50, 100, 200, 300 μM) for 24 h. (a) Chemical structure of Nef. (b) Effect of Nef treatment on WERI-Rb-1 cells viability by CCK-8 assay. "Neferine 0 μM" means "control". Data are expressed as mean ± SD of three independent experiments (*$P < 0.05$ vs. control; **$P < 0.01$ vs. control). (A color version of this figure is available in the online journal.)

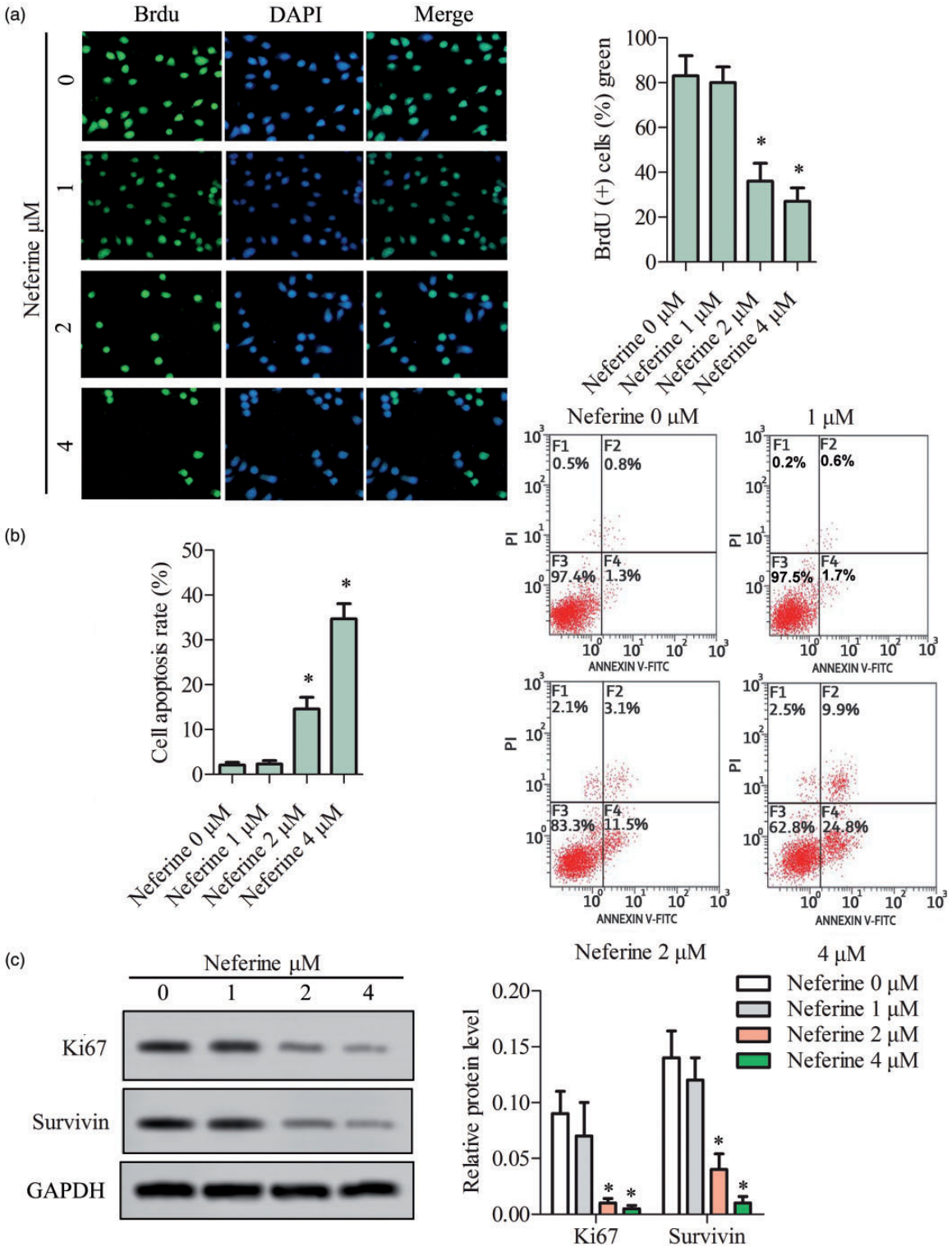

Figure 2. Nef treatment inhibited proliferation of WERI-Rb-1 cells. (a) BRDU staining detected proliferation. Scale bars represent 50 μM. (b) Flow cytometry for the determination of apoptosis. (c) Western blot assay for the expression levels of marked proteins (Ki67 and Survivin). "Neferine 0 μM" means "control". Data are expressed as mean ± SD of three independent experiments (*$P < 0.05$ vs. control; **$P < 0.01$ vs. control).

Discussion

Due to the strong side effects of chemotherapy, natural product therapy has become a new strategy for adjuvant treatment of cancer and other diseases. Recently, a large body of research demonstrated that traditional Chinese medicine can not only prevent and reduce the toxicity and side effects of chemotherapy, but also improve the disease resistance of various cancer patients and help kill cancer cells.[19,20] The discovery of natural products with antitumor activity is invaluable for chemoprevention and cancer treatment.

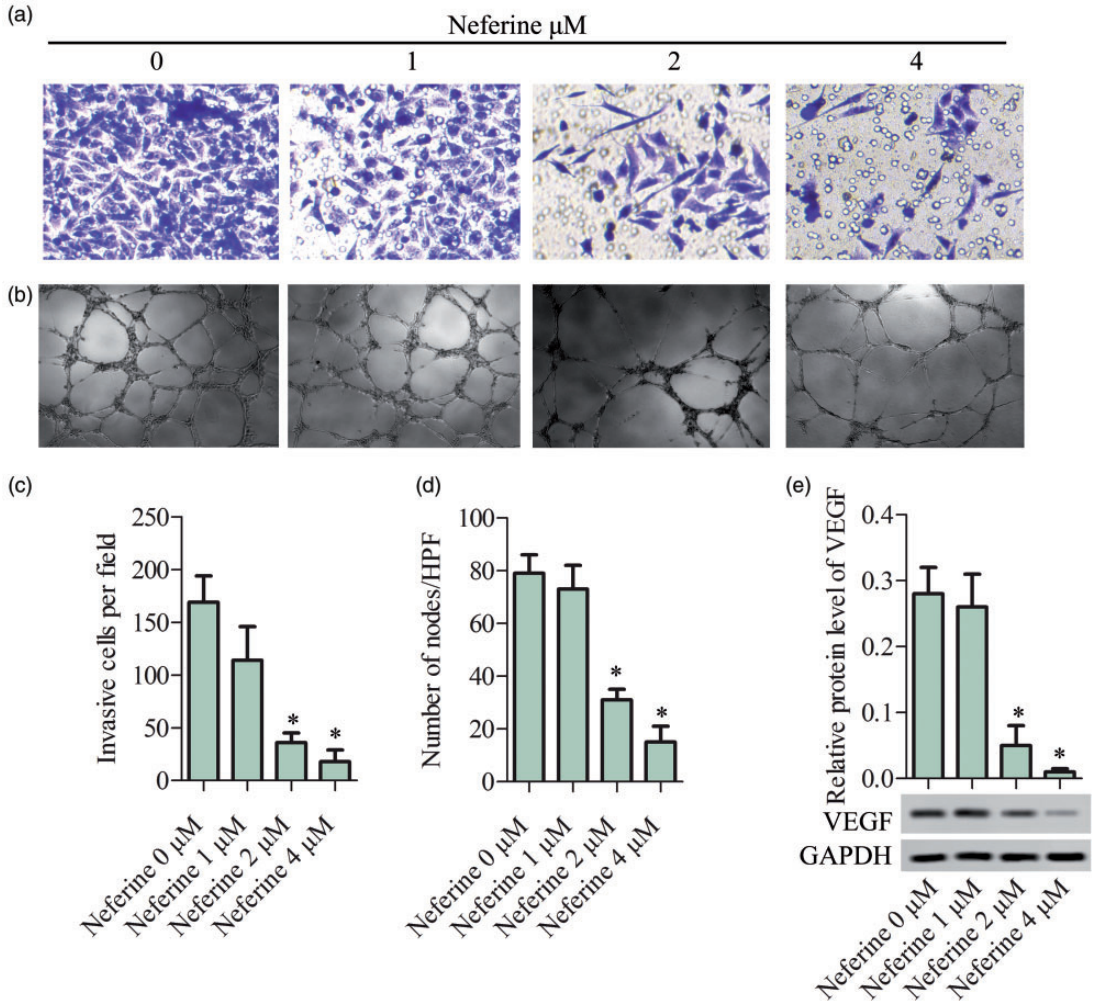

Figure 3. Nef treatment attenuated the invasive ability of WERI-Rb-1 cells. (a) Transwell assay detected invasion of WERI-Rb-1 cells. (b) Ultrastructural changes of microtubules in WERI-Rb-1 cells. (c) Invasive cells per field. (d) Number of nodes/HPF in microtubule formation experiment. (e) Western blot assay for relative protein level of VEGF. "Neferine 0 μM" means "control". Data are expressed as mean ± SD of three independent experiments (*$P < 0.05$ vs. control; **$P < 0.01$ vs. control).

The anticancer activities of phytochemicals were achieved by regulating multiple cellular signaling pathways, such as inflammation, cell survival, metastasis, autophagy, and apoptosis. Alkaloids are special ingredients of Chinese herbal medicine. A series of studies have shown that alkaloids partially isolated from Chinese herbal medicines have antitumor effects, such as vincristine[21] and Nef.[22] In our study, the effects of Nef on retinoblastoma *in vitro* and the oncogenesis *in vivo* were studied. The results suggested that Nef, among 0–4 μM, had no toxic effect on WERI-Rb-1 cells and suppressed proliferation, impaired metastasis, and expedited apoptosis of WERI-Rb-1 cell line *in vitro*. Simultaneously, we found that treatment of Nef induced oxidative stress and mitochondrial membrane potential change in WERI-Rb-1 cell line. In xenograft nude mouse model, Nef treatment dramatically reduced tumor volume and weight and slightly lost weight. These results showed that Nef exerted antiproliferative and anti-invasive activities on retinoblastoma *in vitro* and the oncogenesis *in vivo*, the possible mechanism maybe associated with mitochondrial dysfunction.

Nelumbo nucifera, is a well-known traditional medicinal plant that contains a variety of biologically active compounds.[23] Nef is a dominating bisbenzylisoquinoline alkaloid, exerts pathological effects in a variety of diseases,[24,25] and exists in *Nelumbo nucifera*. Nef was reported to possess cytotoxicity in a dose-dependent manner in primary CML cells.[13,26] In term of cancers, Jun *et al.*[26] pointed out that alkaloid-rich fraction (ARF) from *Nelumbo nucifera* targets vascular smooth muscle cell (VSMC) proliferation and migration to suppress neo-intima formation followed by restenosis in balloon-injured rat carotid artery. Li *et al.*[27] found that Nef can suppress proliferation of vascular smooth muscle cells stimulated by angiotensin II through upregulation of heme oxygenase-1. Zhang *et al.*[22] found that Nef exerted strong growth-inhibitory effect for human osteosarcoma cells, which hinted a direct antitumor effect of Nef. Therefore, they predicted that consuming Nef may have benefits in preventing and treating cancer. These results were consistent with our findings. In the present study, the optimal concentration range of Nef for WERI-Rb-1 cell line was screened at the beginning.

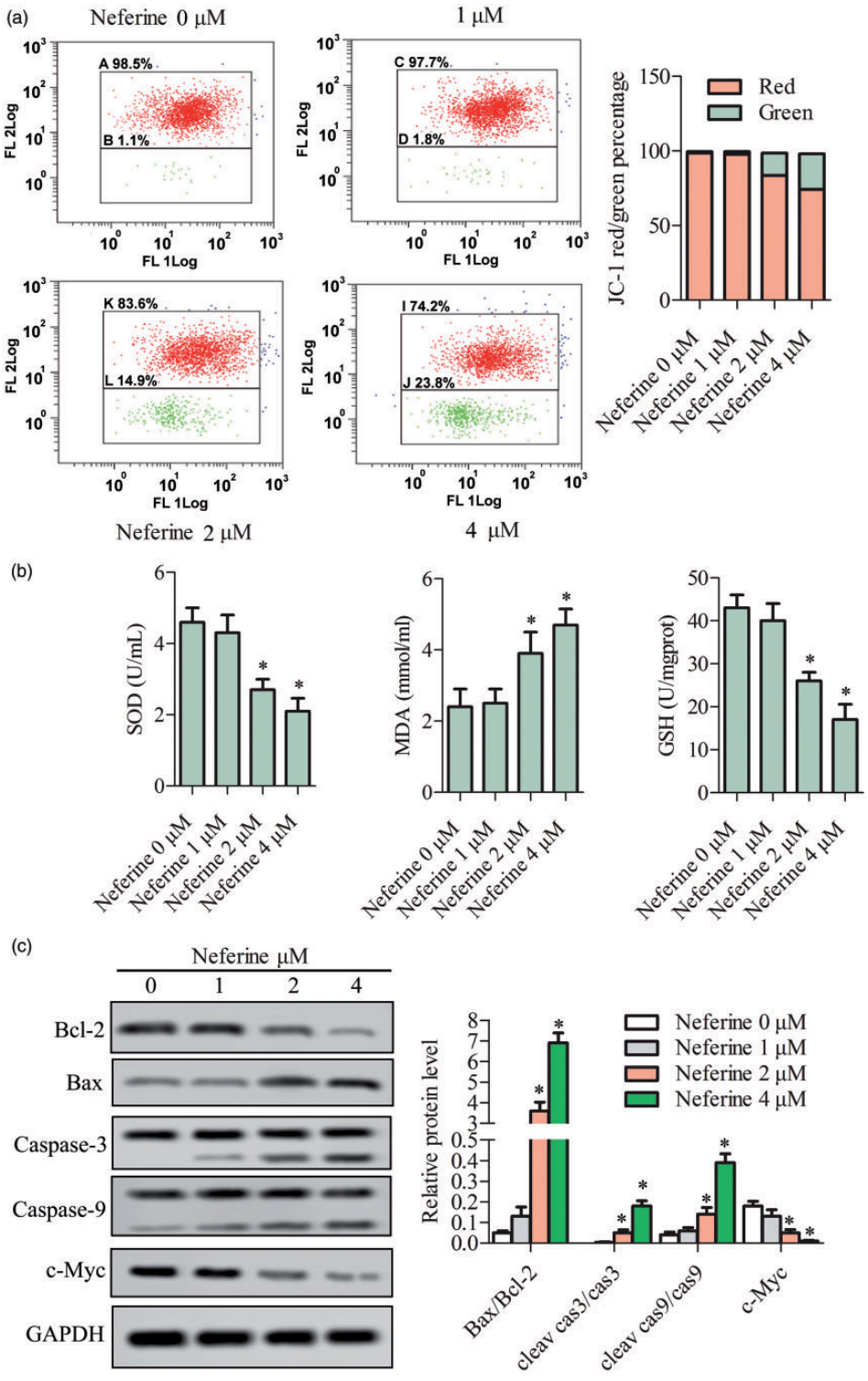

Figure 4. Nef treatment induced mitochondrial dysfunction and WERI-Rb-1 cells apoptosis. (a) Nef treatment induced mitochondrial membrane potential change was evaluated by JC-/1 staining. (b) Oxidative stress indicators (SOD, MDA and GSH) were detected by kit. (c) Western blot assay for the expression of apoptosis marker proteins (Bcl-2, Bax, Caspase-3, Caspase-9, cleaved Caspase-3, cleaved Caspase-9, and c-Myc). "Neferine 0 μM" means "control". Data are expressed as mean ± SD of three independent experiments (*$P < 0.05$ vs. control; **$P < 0.01$ vs. control).

We found that Nef also exerted cytotoxicity on WERI-Rb-1 cell line in a dose-dependent manner. Finally, the concentration range of 0–4 μM was considered to be a reasonable interval and four concentrations (0, 1, 2, 4 μM) were used in subsequent experiments. The results suggested that Nef not only inhibited the proliferation of WERI-Rb-1 cell but also expedited apoptosis of it.

Tumor cell migration is the main reason for the rapid spread of cancer, of which infiltration plays a key role. During the infiltration process, tumor cells first lose their

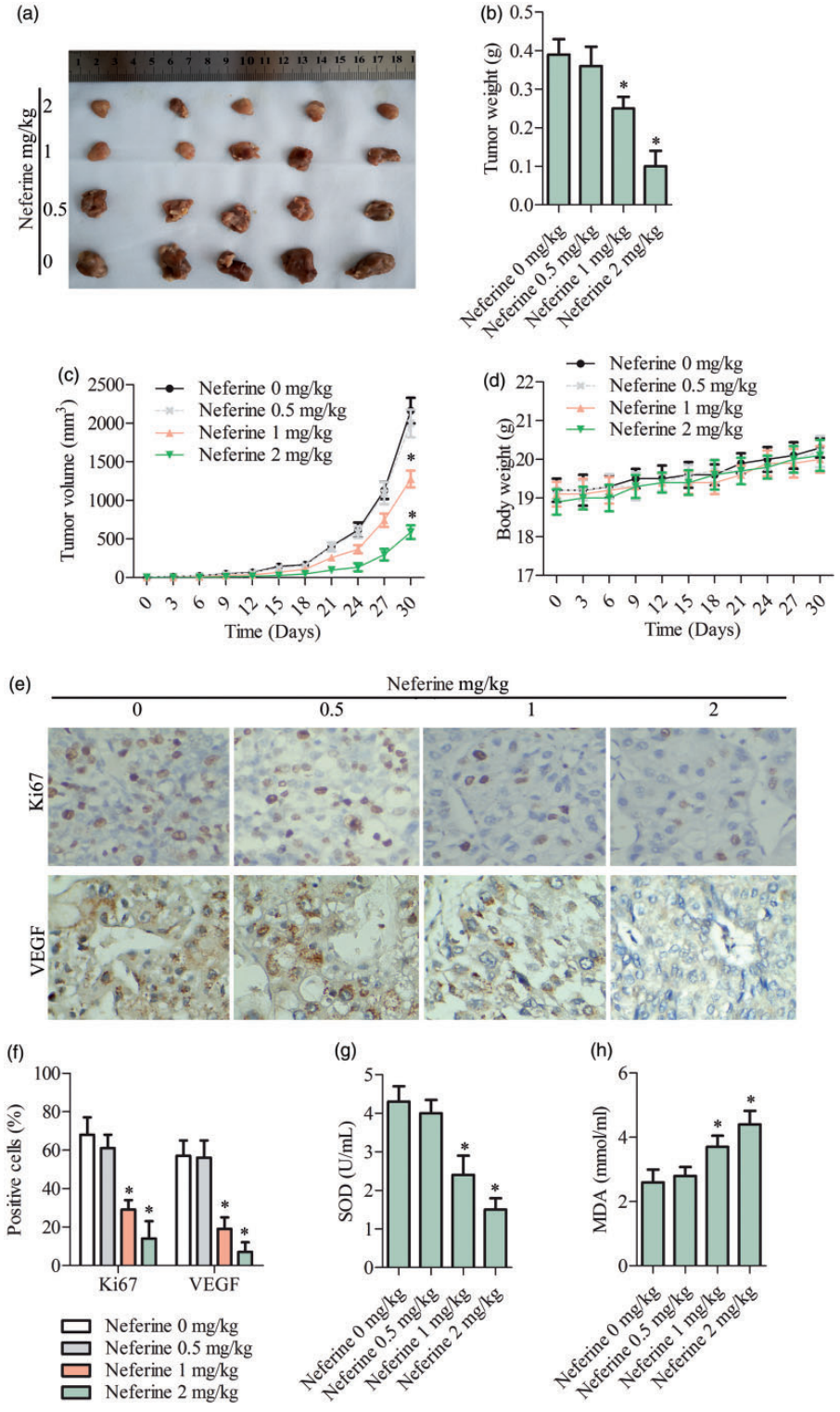

Figure 5. Nef treatment suppressed tumors formation *in vivo*. (a) Tumor tissue. (b) Tumor weight. (c) Tumor volume. (d) Body weight. (e) Immunohistochemistry typical images of Ki67 and VEGF in tumor tissue. Scale bars represent 50 μM. (f) Percentage of Ki67 and VEGF expression in positive cells. (g) The content of SOD. (h) The content of MDA."Neferine 0 mg/kg" means "control". Data are expressed as mean ± SD of three independent experiments (*$P < 0.05$ vs. control; **$P < 0.01$ vs. control).

intercellular connections, then degrade, reshape, and adhere to the surrounding extracellular matrix (ECM), and finally migrate through the ECM to distant places. Therefore, inhibiting tumor cell migration plays an indispensable role in alleviating the development of cancer. Xue *et al.*[28] reported that Nef can suppressed proliferation and migration of GIST-T1 cell line of gastrointestinal stromal tumor by the up-regulation of miR-449a. Manogaran *et al.*[29] reported that anti-cancer activity of Nef was achieved through enhanced activation of MAP kinases, ROS generation, followed by induction of apoptotic and autophagy cell death. In this study, we found that Nef

treatment prevented invasion, inhibited angiogenesis, and reduced vascular endothelial growth factor expression of WERI-Rb-1 cell. These results indicated that Nef possess a good inhibitory effect on tumor cell migration.

Evidences revealed that pharmacological effects of Nef are closely related to mitochondrial function. Particularly, Paramasivan *et al.*[18] found that Nef induced ROS generation mediated by mitochondrial and further caused HepG2 cells apoptosis of caspase-dependent. Some scientists have turned the direction of cancer cell apoptosis to mitochondrial fission.[30] It is well known that the most common cause of mitochondrial dysfunction is oxidative stress.[31] *In vitro* experiments of this study, Nef treatment not only decreased SOD and GSH production, enhanced MDA leakage, but also promoted JC-1 red/green percentage. These results indicated that Nef treatment induced oxidative stress and further triggered mitochondrial dysfunction. *In vivo* experiments of this study, Nef treatment decreased SOD production and enhanced MDA leakage, simultaneously, diminished the percentage of positive cells expressed Ki67 and VEGF dramatically. These results may demonstrate that Nef treatment induced oxidative stress and accelerated tissue apoptosis in cancer tissue. Therefore, we predicted that Nef treatment inhibited retinoblastoma and oncogenesis *in vivo* by inducing mitochondrial dysfunction. Fortunately, our results were consistent with the previous study. Cheng *et al.*[32] reported that Piscidin-1 induced apoptosis via mitochondrial reactive oxygen species-regulated mitochondrial dysfunction in human osteosarcoma cells.[32] Nevertheless, key steps remain to be corroborated, especially those that lead to selective killing of cancer cells. How to effectively use mitochondrial dysfunction and its regulatory role in cancer to kill anti-cancer cells in a heterogeneous cell population in tumors is still a promising but not yet fulfilled premise.

In conclusion, Nef could inhibit proliferative capacity, invasion and microtubule forming abilities of WERI-Rb-1 cell line; moreover, Nef could induced oxidative stress and further caused mitochondrial dysfunction *in vitro*. Besides, Nef inhibited tumors weight and volume in nude mice model and selectively inhibited proliferative capacity, invasion, and microtubule forming abilities of WERI-Rb-1 cell line *in vivo*. The main mechanism maybe involved in mitochondrial dysfunction triggered by Nef treatment induced oxidative stress. Thence, Nef might be a promising retinoblastoma therapeutic.

Authors' contributions: Qiuming Li and Jing Wang participated in the design of this study, Jing Wang and Yanmin Dong performed the experiments, Jing Wang analyzed the data and wrote the manuscript. The final manuscript read and approved by all authors.

DECLARATION OF CONFLICTING INTERESTS

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