The expanding prevalence of cancer in recent years and its influence on different aspects of day to day life has evolved it into one of the biggest predicaments of the century (1). According to estimates, cancer-related mortality occurs in a global population close to 7 million. It is projected that the rise of fresh cancer incidences will rise from 10 to 15 million will take place around 2020 (2, 3). Meanwhile, breast cancer is considered amongst the most predominant type of malignant neoplasms in women (4) and around one million new cases are registered annually (5). One of the most frequently diagnosed cancer among the Chinese female population is breast cancer (6). The burden of female breast cancer has undergone rapid growth over the past decade (7). Advances in the socioeconomic status, westernized food habits and lifestyles, obesity, reproductive and endocrine aspects are a few of the significant risk factors accounting for the emergence of female breast cancer, somewhat explaining the higher incidences and mortality rates in urban areas as compared to rural ones (8). Breast cancer is the most prevalent malignancy and is the predominant cause of mortality among women aged 45-55 years (9). It is essentially a carcinoma of the tissue involving the inner layer of milk glands or lobules, and ducts (10). The preliminary risk factors being age (11), increased hormonal levels (12), race, socioeconomic status, and deficiency of iodine in the regular diet (13-15). Every 1-in 8 women suffer from breast cancer, necessitating complete tissue removal, chemotherapy, radiotherapy, and hormone therapy in most cases (16). Despite the continuous deployment of novel treatment schemes and agents over the decades, most cancers still suffer a relapse and remain fatal. Cancer cells, through their access to the huge data about the human genome, have an amazing ability to organize adaptive strategies for even the most versatile treatments (17). Thus, the insufficient proficiency of the orthodox treatments to eradicate the disease motivates us to identify alternate chemotherapeutic agents.

A few non-nutritive phytochemicals found in natural products comprising of pharmacological properties confirm that they restrain, delay and/or converse malignancies evoked by either environmental constituents or lifestyle. Among these non-nutritive phytochemicals, flavonoids are one such family, endowed with robust antioxidant properties depending upon their ability to delocalize unpaired electrons and the existence of hydrogen and electron-donating groups correlated with their potential
of coordinating metal ions, consequently inhibiting DNA damage and performing as a chemotherapeutic agent (18, 19). Rutin (3,3’4’,5,7-pentahydroxyflavone-3-rhamnoside), a flavonol, is found in large quantities in passionflower, buckwheat, tea, and apple and possess a variety of pharmacological activities, like antioxidant, cytoprotective, vasoprotective, anticarcinogenic, neuroprotective and cardioprotective (20-26). Rutin was also reported to stimulate apoptosis in human breast cancer cells, whereas reinstating the chemosensitivity of HER2 negative and triple-negative breast cancer cells (27). Elsayed et al reported that rutin prevents the activity c-Met kinase in TNBC cells thus considerably reducing the growth of TNBC MDA-MB-231/GFP orthotopic xenografts in murine models (28).

Organometallic compounds centered around transition metals have developed as favorable scaffolds in the quest for antitumor leads (29). Organometallic complexes display a variety of structural assortments of different geometrical shapes subjected to the oxidation state of the metal center and modification of their co-ligands (30). Vanadium, a transition metal, is known to possess a variety of biological roles in anticancer, antidiabetic and anti-infective diseases (31). Erswhile research report that vanadium plays an important role in xenografted carcinomas of liver, lung, breast and gastrointestinal tract and human cell lines. Vanadium is also considered to be involved in the repression of cell proliferation and initiation of apoptosis along with the interruption cell cycle in chemically induced murine models of mammary carcinogenesis and breast cancer cell lines (32, 33).

Recently the development of metal-based anticancer complexes has engrossed substantial consideration in cancer research. Metal complexes with flavonoids have been reported to influence antioxidant, anti-tumor, anti-microbial, anti-inflammatory, and insulin-mimetic activities (34-36). Roy and his team investigated the chelation property of rutin with vanadium and reported that the complex was capable of reacting with free radicals or terminate chain reactions in time and dose-depended manner. They also reported the toxicity of vanadium-rutin complex in balb/c mice (37). However, to the best of our knowledge, the chemopreventive activity of vanadium-rutin complex in breast cancer and the underlying mechanism has not been investigated to date. Our current study aims to investigate the chemopreventive effect of the vanadium-rutin complex in human breast cancer cell lines.

**EXPERIMENTAL**

All reagents used for experimental purpose were of analytical reagent grade. Methanol, vanadium (IV) oxide sulfate monohydrate, Rutin trihydrate, highly polymerized CT-DNA (calf thymus DNA), Tris–HCl, fetal bovine serum (FBS), insulin L-glutamine, sodium pyruvate, streptomycin, penicillin, MTT (3-(4,5-dimethyl thiazole-2-yl)-2,5-diphenyltetrazolium bromide), Annexin V and propidium iodide (PI) were purchased from Sigma Aldrich Chemical Co (St. Louis, MO). Anti-human p53, Bax, Bcl2, and VEGF were purchased from AnaSpec Inc. (San Jose, CA). The Michigan Cancer Foundation-7 (MCF-7) breast cancer cell line and MDA-MB-231 triple-negative (TNBC) cell lines were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). Other reagents used for the experimental purpose were obtained in the purest form from local firms.

**Synthesis of the vanadium–rutin complex**

The complex was synthesized as per the methods of Roy and team (37). Rutin trihydrate was added dropwise to a saturated solution of VOSO4•5H2O and stirred continuously. The pH was adjusted to 6.0 with 1molar sulphuric acid. The precipitated green solid was filtered through a fritted glass funnel and washed several times with water. The final product was kept over anhydrous CaCl2 to obtain a dry powder.

**DNA binding study of vanadium-rutin complex with CT-DNA**

Electronic absorption titration

The mode of intercalation of calf thymus DNA by vanadium-rutin complex was measured by UV-Visible spectra using UV-1800 Shimadzu double beam spectrophotometer. Solution of the CT-DNA and vanadium-rutin complex was scanned using a 0.5 cm (1 mL) quartz cell. Spectra were recorded by progressive addition of pure DNA to the vanadium-rutin complex (38). The stock solution of DNA (of concentration 1 × 10⁻³ M per nucleotide) was prepared at pH 7 by dissolving DNA in 5 mM Tris-HCl buffer and 50 mM NaCl. A solution of CT-DNA gave a ratio of about 1.8-1.9 : 1 at 260-280 nm wavelength, which indicated that the DNA was free of protein.

**CT-DNA cleavage by agarose gel electrophoresis**

The DNA cleavage activity of the vanadium-rutin was monitored by agarose gel electrophoresis on CT-DNA. Each reaction mixture contained
30 µM of CT-DNA, 10, 20 and 30 µM of complex in 50 mM Tris–HCl, (pH 7.1). The reaction was incubated at 37°C for 2 h. After incubation, 1 µL of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol and 60% glycerol) was added to the reaction mixture and loaded onto a 1% agarose gel containing 1.0 µg/mL of EB (3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide). The electrophoresis was performed for 2 h at 50 V in Tris–acetic acid–EDTA (ethylenediaminetetraacetic acid) buffer, pH 7.1. The bands were visualized under UV light and photographed.

**In vitro experiments**

**Cell culture**

The cell lines were routinely maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS containing antibiotics, penicillin/streptomycin (0.5 mL/L) in an atmosphere of 5% CO₂ and 95% air at 37°C.

**Cell viability assay**

The assay was determined by metabolizing yellow tetrazolium salts MTT (3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into reduced formazan crystals by mitochondrial succinate dehydrogenase (39). The MCF-7 and MDA-MB-231 cells were plated in 96-well plate with 5.0 × 10³ cells per well in culture medium and incubated overnight at 37°C in 5% CO₂ humidified incubator and were subsequently treated with five different concentrations (20, 50, 100, 200 and 400 µM) of vanadium-rutin complex for 12, 24 and 48 h in a complete growth medium. The medium was removed after treatment, and MTT solution (0.5 mg/mL) was added to each well and incubated at 37°C for 3 h. The optical density of solubilized crystals in DMSO was measured at 560 nm on a microplate reader. The percentage of cell viability was calculated using the following equation.

\[
\text{% viability} = 100 - \% \text{ of cytotoxicity}
\]

**DNA fragmentation**

The MCF-7 and MDA-MB-231 cells (5.0 × 10³ cells per well) cultured in a 96-well plate were treated with different concentrations (25, 50, and 100 µM) and (50, 100 and 150 µM) of vanadium-rutin complex and DMSO control for 48 h. The cells were assessed for nuclear blebbing and chromatin condensation by staining them with fluorescent nuclear dye DAPI according to the method followed by Roy et al (40).

**Cell cycle and detection of apoptosis by flow cytometry**

For the determination of cell cycle phase distribution, MCF-7 and MDA-MB-231 cells cultured in a 96-well plate were suspended and permeabilized then their nuclear DNA was labeled with propidium iodide (PI). Cell cycle phase distribution was determined on FACS (fluorescence-activated cell sorter). A total of 10,000 events was acquired and analysis of flow cytometry data was performed using ModFit software. To distinguish between apoptosis and necrosis, PI and fluorescent-tagged Annexin-V were added directly to the medium of both untreated and vanadium–rutin treated MCF-7 and MDA-MB-231 cells (5.0 × 10³ cells per well) after 48 h of vanadium–rutin treatment. The concentrations of vanadium–rutin used were 25, 50, and 100 µM and 50, 100 and 150 µM respectively. The mixture was incubated for 15 min at 37°C. Excess PI and Annexin-V were then washed off and analyzed by flow-cytometer using Cell Quest software. Electronic compensation of the instrument was done to exclude overlapping of the emission spectra. A total of 10,000 events were acquired and the cells were properly gated for analysis.

**Detection of p53, Bax, Bcl2, and VEGF protein expression by western blot analysis**

The expressions of p53, Bax, Bcl2, and VEGF were detected in MCF-7 and MDA-MB-231 cells by western blot analysis. The MCF-7 and MDA-MB-231 cells were cultured in a T75 cm² flask at a density of 1 x 10⁶ cells/mL and incubated in CO₂ incubator overnight at 37°C for 48 h to get 80-90% confluency. The spent medium was aspirated and MCF-7 cells were treated with the complex at doses 25, 50, and 100 µM and MDA-MB-231 cells were treated with the complex at doses 50, 100 and 150 µM. At the end of 48 h, the medium was removed from all the wells and washed with PBS. PBS was removed and 2000 µL of the trypsin-EDTA solution was added followed by incubation at
37°C for 3-4 min. 5 mL culture medium was added and cells were harvested directly into 12 × 75 mm polystyrene tubes. Total cell lysates at approximately 2 × 10⁶ – 1 × 10⁷ cells per mL were prepared and the amount of protein in each sample was measured by Bradford Assay. The cell extracts were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10–12%) followed by transfer to polyvinylidene difluoride membranes and blocked with Tris buffer (25 mM) containing 0.15 M of NaCl, 0.1% Tween 20 with 2-5% nonfat dry milk. The membranes were incubated with p53, Bax, Bcl2, and VEGF primary antibodies at 4°C for 2 h, rinsed in PBS and incubated with horseradish peroxidase-labeled secondary antibody for 1 h. The membranes were rinsed for 30-60 min with five or more changes in blotting buffer. Chemiluminescent (ECL western blot analysis) kit was used to detect the protein loading against β-actin as control (41).

RESULTS

The obtained complex was characterized by Fourier transform infrared analysis, UV–Visible spectroscopy, Mass spectrometry analysis, Nuclear magnetic resonance spectroscopy and Scanning Electron Microscopy in accordance with the approaches described by Roy and team. The final structure of the complex has been denoted in Figure 1 (37).

DNA binding study of vanadium-rutin complex

Electronic absorption titration

The addition of the CT-DNA to the complex showed a significant decrease in the absorbance of the vanadium-rutin complex (Fig. 2A). The decrease in the absorption of the resulting solution could be attributed to the alteration of the structure of DNA double helix after binding with the complex. The decrease in the absorption spectra of the complex with the increasing concentrations of DNA indicated towards the non-intercalative mode such as outside binding of the complex with the CT-DNA.

Gel electrophoresis

DNA cleavage is characterized by the relaxation of supercoiled form into nicked circular and linear forms. The cleaving efficacy of the complex in CT-DNA was estimated by agarose gel electrophoresis. Different complexes cleave CT-DNA in various forms of efficacy depending upon the binding affinities of complexes to the DNA (42). As shown in Figure 2B, the absence of complexes (lane 1), no distinct DNA cleavage was observed, however, the interactions of the complex and CT-DNA show the efficacy of the complex in changing the mobility and the shape of CT-DNA. The results suggested that the vanadium-rutin complex caused damage to the DNA.
In-vitro studies

Vanadium-rutin complex sensitizes breast cancer cells via modulation of...

Vanadium-rutin complex inhibits cell viability

The effect of the vanadium rutin complex on the viability of MCF-7 and MDA-MB-231 cells is depicted in Figures 3A and B. A dose and incubation time-dependent inhibitory effect was noted on the viability of both MCF-7 and MDA-MB-231 cells following treatment with vanadium-rutin complex. The cell viability of the complex against MCF-7 cells at 20, 50, 100, 200, and 400 µM concentrations was found to be 99, 79, 68, 54, 43, and 36 respectively, after 48 h and cell viability of the complex against MDA-MB-231 cells at 20, 50, 100, 200 and 400 µM concentrations was found to be 97, 86, 73, 62, 49 and 42 respectively. The highest inhibition rate was found to be 64% at 400 µM in MCF-7 cells and 58% at 400 µM concentration of vanadium-rutin complex in MDA-MB-231 cells at 48 h.

Vanadium-rutin complex causes apoptosis

Nuclear blebbing and chromatin condensation in MCF-7 and MDA-MB-231 cells were detected by using DAPI dye. Cells containing condensed chromatin morphologically signify cells undergoing apoptosis and fluoresce with bright blue color. The MCF-7 and MDA-MB-231 cells were treated with 25, 50, and 100 µM and 50, 100 and 150 µM of the complex for 48 h to induce apoptosis. Figures 4A and B show that the complex induces nuclear condensation in a dose-dependent manner. It was
observed that the cells treated with 400 µM concentration of the complex showed maximum chromatin condensation and signifies the highest expanse of apoptosis in both MCF-7 and MDA-MB-231 cells.

**DNA fragmentation**

A significant feature of apoptosis is DNA fragmentation and dose-dependent induction of apoptosis was again observed in both MCF-7 and MDA-MB-231 cells treated vanadium-rutin complex consisting of a smear pattern of DNA fragmented with both low and high molecular weight DNA fragments as compared to the ladder (Fig. 4C and D). The control treatment consisted of exposure to 0.1% DMSO for 48 h and showed clear bands of intact DNA and the smear pattern of damaged DNA.

![Figure 5](image)

**Figure 5.** Detection of apoptosis in (A) MCF7 and (B) MDA-MB-231 cells by flow cytometry. In a double-labeled system, the unfixed cells, both vanadium rutin-treated and control were stained with PI and Annexin-V and analyzed by flow cytometry. Dual parameter dot plot of Annexin-V (x-axis) and PI (y-axis) were shown. Quadrants: lower left (LL) – live cells, lower right (LR) – early apoptotic cells, upper right (UR) – late apoptotic cells, upper left (UL) – necrotic cells. Percentage of apoptotic cells versus concentration in (C) MCF7 and (D) MDA-MB-231 cells. Three independent experiments were performed, *P < 0.01, significant difference between complex treatment and control.

![Figure 6](image)

**Figure 6.** Analysis of cell cycle phase distribution of (A) MCF7 and (B) MDA-MB-231 cells after the treatment with vanadium-rutin complex for 48 h by single label flow cytometry. The cells were fixed and DNA was labeled with PI. The histogram data represents the DNA content (x-axis) versus cell number (y-axis). Data is representative of three different experiments. Quantitative distribution of the percentage of (C) MCF7 and (D) MDA-MB-231 cells in different phases of the cell cycle.
Effect of vanadium-rutin complex in apoptosis induction by flow cytometry and cell cycle analysis

MCF-7 and MDA-MB-231 undergoing apoptosis were observed by staining them with Annexin-V and PI by treating them with 25, 50, and 100 µM and 50, 100 and 150 µM concentrations respectively of the complex for 48 h. Flow cytometry analysis can distinguish stained cells into four categories, namely viable (Annexin-V-PI-), early apoptosis (Annexin-V+PI-), late apoptosis (Annexin-V+PI+) and necrotic (Annexin-V-PI+) cells. Figures 5A and B demonstrate the distribution of cells undergoing treatment with different concentrations of the complex after 48 h. Figures 5C and D show that the percentages of apoptotic cell are 16.25, 25.91 and 37.54% following treatment with 25, 50, and 100 µM of the complex in MCF-7 cells and 10.28, 31.95 and 44.3% following treatment with 50, 100 and 150 µM of the complex in MDA-MB-231 cells as compared to control (p < 0.01). Furthermore, a dose-dependent increase in the populations of early apoptotic cells was also observed in the cells following treatment with the complex after 48 h.

Flow cytometry was also used to investigate the distribution of cell cycle phases with cellular DNA content. The apoptotic cells in the cell cycle histogram are represented by the number of sub-diploid cells (Fig. 6A and B). The MCF-7 treated with...
25 µM, 50 µM and 100 µM of the complex denoted 56.7%, 52.3% and 48.7% cells in the G0/G1 phase whereas the G0/G1 phase treated with 50 µM, 100 µM and 150 µM of the complex showed 75.3%, 68.3% and 52.3% cells in the G0/G1 phase. Simultaneously an increase of cells in the S-phase was noted after complex treatment in a dose-dependent manner (Fig. 6C and D). Thus, it can be noted that the complex induces a decrease in the percentage of G0/G1 phase of the cell cycle in a dose-dependent manner in both cell lines. Compared to the treated cells the fraction of cells in MCF-7 cells was G0/G1-63.5%, S-15.8% and G2/M-20.7%. The fraction of cells in each phase of the MDA-MB-231 control is G0/G1-81.5%, S-5.8% and G2/M-12.7% respectively.

Effect on the expressions of p53, Bax, Bcl2, and VEGF with vanadium-rutin treatment.

The expression of proteins like p53, Bax, Bcl2, and VEGF was studied by Western blot analysis of MCF-7 and MDA-MB-231 cells followed by treatment with different concentrations of vanadium-rutin complex for 48 h (Fig. 7A and B). A dose-dependent downregulation of Bcl2 and VEGF were noted after treatment with 25, 50, and 100 µM (MCF-7) and 50, 100 and 150 µM (MDA-MB-231) of the complex for 48 h. Subsequently, a distinguished increase in the expressions of p53 and Bax was noted in MCF-7 and MDA-MB-231 cells treated with 100 µM and 150 µM concentration of the complex.

DISCUSSION

Flavonoids are natural products that present an appealing source of diverse structures that are capable of demonstrating potnet biological and pharmacological activities (43). Additionally, flavonoids are capable of forming complexes with metal ions possessing better pharmacological activities than the parent flavonoids (44). Metals of the transition group exhibit various oxidation states and can interact with negatively charged molecules to produce pharmacologically active complexes (31). Vanadium, a transition metal, is a biologically important element incorporated in a number of physiological processes. Vanadium compounds, in particular organic derivatives, are effective in various diseases like diabetes, cancer, hypertension, Ischemia and Myocardial Stroke (45). The formation of a complex between metals and flavonoids plays an important role to forage free radicals as compared to the parent molecule resulting in reduced toxicity of the metals (46).

Our current study encompasses the synthesis of vanadium-rutin complex and evaluating its anticarcinogenic property in human breast cancer cells. The interaction of the complex with CT-DNA has been studied by DNA gel electrophoresis as well as spectroscopic methods. The decrease in the absorption of the DNA complex system with increasing concentration of the DNA denotes that the complex binds to DNA by non-intercalative mode. The DNA gel electrophoresis displayed a smear pattern of DNA fragmentation with high and low molecular weight bands of cleaved DNA, emphasizing that the complex is capable of inducing DNA damage.

The results acquired from the cell viability studies and oligonucleosomal fragmentation by DAPI staining indicated that the vanadium-rutin complex diminishes cell proliferation and encourages apoptosis both MCF-7 and MDA-MB-231 cells. One of the fundamental characteristics of anti-cancer agents is their capability to regulate cell cycle and which is achieved through the arrest of G1 and G2 phases (47). The style of apoptosis induced by vanadium-rutin complex was established by flow cytometric analysis using FITC tagged Annexin-V and PI staining. The results indicated that the vanadium-rutin complex instigates a higher percentage of early apoptosis in both MCF-7 and MDA-MB-231 cells along with a decrease in the percentage of G0/G1 phase of the cell cycle in a dose-dependent manner in both cell lines.

For an elaborate comprehension of the mechanism of action of vanadium-rutin complex, a cell-based reporter assay was conducted to study the effect of the complex on p53, Bax, Bcl2 and VEGF signaling pathways. p53 is the most commonly mutated gene in human cancers (48). It has been noted that the mutations of p53, play a central role in the evolution of tumor pathological process throughout the development of adenoma-carcinoma (49) MDA-MB-231 cells have a mutant p53 whereas MCF-7 possesses the wild type. Although the primary purpose of the p53 mutations is to contravene the tumor suppressor role of wild type p53, there is considerable evidence that the resulting mutant p53 protein may also lead to tumorigenesis by means of a ‘gain-of-function’ mechanism (50). The ‘gain-of-function’ theory suggests that p53 mutations actively promote tumourigenesis, independent of the loss of wild type p53 function. Triggered PI3K’s catalyze PIP3 formation from PIP2, and lipid phosphatase PTEN (phosphatase and tensin homolog) specifically inhibits PI3 K activity by de-phosphorylating PIP3 into PIP2, thus serving as the main negative PI3 K regulator. Activated PI3 further assists
Vanadium-rutin complex sensitizes breast cancer cells via modulation of... 97

the phosphorylation of Akt and mTOR pathways which have a crucial role in tumor progression (51). Thus activated, mTOR also promotes angiogenic factors like VEGF thus indirectly backing up tumor growth in breast cancers (52). Our results denote an increase of p53 expression and a decrease in VEGF expressions leading us to conclude that the vanadium-rutin complex is capable of modulating these pathways in the regression of breast cancer.

Apoptosis, the hallmark of cancer cells, ensues in two different pathways namely extrinsic and intrinsic. The intrinsic pathway occurs around mitochondria and Bcl2 is one of the key factors involved in this pathway, which interacts with Bax bringing an alteration of the mitochondrial membrane potential directing the discharge of regulatory proteins to activate the cellular caspase cascade (52). Bcl2 a membrane-associated protein, possesses anti-apoptotic and anti-oxidative properties, thus portraying a pivotal role in stimulating cell survival and barring the actions of pro-apoptotic proteins like Bax. The occurrence of cancer may be traced to the over-expression of anti-apoptotic genes and under-expression of pro-apoptotic genes. The over-expression of anti-apoptotic proteins assists in defining mitochondrial integrity and helps to releases effector proteins that halt apoptosis thus allowing cellular proliferation to occur (53). In contrast, the expression of pro-apoptotic proteins like Bax is considerably inhibited by oncogenic kinases (54). The observations from the current study are comparable to the experiments conducted by Roy and the team while examining the effects of vanadium-quercetin (an aglycone of rutin) complex on MCF-7 cells. Roy’s study denoted that the vanadium-quercetin complex was capable of inducing cell cycle arrest and apoptosis in a dose-dependent manner in MCF-7 cells, also the complex in a similar fashion upregulated p53 and Bax with downregulation of Bcl2 and VEGF (55). Our in-vitro study displayed that complex treatment significantly decreased the Bcl2 expression, whereas the expression of Bax was found to significantly increase in the MCF-7 and MDA-MB-231 cells (Fig. 8).

CONCLUSION

Taken together, our work uncovered a novel molecular mechanism of vanadium-rutin complex responsible for the p53 mediated apoptosis in the breast cancer cells. Apoptosis commences by the activation of the intrinsic apoptotic pathway regulated by Bcl2 on the proapoptotic Bax, by the steady retrotranslocation of Bax from the mitochondrial outer membrane to the cytosol. Besides, the complex can also exert antiangiogenic activity by downregulating the VEGF proteins. The in vitro results provide substantial evidence that low doses of vanadium-rutin chemotherapy could interrupt the progression of breast cancer by regulating intrinsic apoptosis as well as antiangiogenic pathway, thus aiming to be a potential candidate for cancer therapy in clinical settings.

Conflicts of interest

The authors declare no conflict of interest in regard to this study.

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98 ZHU HAIFENG et al.


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