

ORIGINAL ARTICLE

**ASSESSMENT OF GENOTOXICITY OF VINCRIStINE,
VINBLASTINE AND VINOReLBINE IN HUMAN CULTURED
LYMPHOCYTES: A COMPARATIVE STUDY**

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ABSTRACT

Vincristine (VCR), vinblastine (VBL) and vinorelbine (VRL) are anticancer agents from the Vinca alkaloid family that have the potential to induce genotoxic effect. The aim of the present study was to compare the genotoxic effect of VCR, VBL and VRL. Levels of 8-hydroxy-2-deoxy guanosine (8-OHdG) and sister chromatid exchanges (SCEs) were measured in cultured human blood lymphocytes treated with VCR, VBL and VRL at concentrations of 0.01 and 0.1 µg/mL. Results showed that VCR, VBL and VRL significantly increased the 8-OHdG levels ($p < 0.05$), whereas it did not cause a significant increase in the frequencies of SCEs in human blood lymphocytes as compared to controls. On the other hand, all three agents significantly increased cells mitotic index ($p < 0.05$). At both tested concen-

trations, the magnitude of the increase in 8-OHdG was VBL>VCR>VRL. In conclusion, VCR, VBL and VRL induce DNA damage as indicated by the increase in the 8-OHdG biomarker but with different magnitude.

Keywords: Chromosomal aberrations; Human blood lymphocyte; 8-hydroxy-2-deoxy guanosine (8-OHdG); Sister chromatid exchanges; Vincristine (VCR); Vinblastine (VBL); Vinorelbine (VRL).

INTRODUCTION

Vinca alkaloids are a subset of drugs derived from the Madagascar periwinkle plant (*Catharanthus roseus*) [1]. They include the natural products vincristine (VCR) and vinblastine (VBL) and the semi-synthetic derivative vinorelbine (VRL). Vinca alkaloids have been used for cancer management [2]. Chemically, vinca alkaloids have dimeric chemical structures composed of two basic multi-ringed units, an indole nucleus (catharanthine) and a dihydroindole nucleus (vindoline), joined together with other complex systems [2,3].

Different Vinca alkaloids have their own unique properties. The VBL inhibits angiogenesis [4]. It is also associated with anti-diuretic hormone secretion and angina, and applied to treat Hodgkin's disease, non-Hodgkin's lymphoma and breast cancer [5]. The VRL showed a significant anti-tumor activity in patients with breast cancer and induces anti-proliferative activity in osteosarcoma [6]. Moreover, VCR has been shown to have a mild myelo-suppressive action

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[7,8]. It is also widely used to treat pediatric leukemias, solid tumors, and hematological malignancies [2]. During cell division, Vinca alkaloids bind to the building blocks of a protein called tubulin, inhibiting its formation, which normally works in cells to create mitotic spindle [9].

Previous studies have shown that Vinca alkaloids have the potential to induce genotoxic effects in different biological systems. The VCR and VBL have been shown to increase the frequency of micronuclei in experimental animals and in cultured human lymphocytes [10-13]. In addition, they have also been shown to cause chromosomal mutations *in vivo* and in cultured cancer cells [14,15]. In cultured human lymphocytes, VCR and VBL increased the rate of micronucleus formation [16]. In *Drosophila*, VCR and VBL induced a significant genotoxic effect as measured using wing somatic mutation and the recombination test [17]. However, some other studies have shown lack of mutagenic effect for Vinca alkaloids *in vivo* and in cultured cells [18-20]. Thus, the genotoxicity of Vinca alkaloids is still controversial. In addition, oxidative DNA damage induced by these compounds has still not been investigated. The aim of the present study was to compare the genotoxic effect of VCR, VBL and VRL on human cultured lymphocytes using 8-hydroxy-2-deoxy guanosine (8-OHdG) and sister chromatid exchanges (SCEs) assays. The 8-OHdG is a marker that reflects oxidative DNA damage, while the SCEs assay reflects genotoxicity induced as a result of breaks in DNA during DNA recombination.

MATERIALS AND METHODS

Subjects. Five healthy male nonsmoking volunteers, with an age range of 20-25 years old, were the blood donors. Exclusion criteria were alcohol, cigarette smoking, medications and vitamin use. A sample of whole venous blood (15 mL) was collected in heparin tubes from each donor under sterile conditions. Whole blood cells were cultured within 1 hour of sampling. Informed consent was obtained from each volunteer. This study was approved by the Institutional Review Board of Jordan University of Science and Technology, Irbid, Jordan.

Drugs and Treatment. Vincristine, VBL and VRL were purchased from Sigma-Aldrich Produktions GmbH (Steinheim am Albuch, Germany).

To evaluate the effect of VCR, VBL and VRL on DNA, seven groups were used: a control group and drug-treated groups (VCR, VBL and VRL; each at concentrations of 0.01 and 0.1 µg/mL). The control group was treated with distilled water. Drug-treated groups were treated with the corresponding drug 4 hours prior to harvesting. This was based on previous studies [21,22].

Lymphocytes Culture. Blood cultures were set up by inoculating 1 mL of freshly drawn blood into 75 mL tissue-culture flasks containing 9 mL of peripheral blood (PB)-Max medium composed of Roswell Park Memorial Institute (RPMI) 1640 medium with 15.0% fetal bovine serum (FBS), 1.0% penicillin-streptomycin and 3.0% of phytohaemagglutinin (Gibco-Invitrogen Ltd., Paisley, Ren-frewshire, UK). To collect metaphase cells, cultures were exposed to 100 µL of 10 µg/mL colcemid (Gibco-Invitrogen Ltd.) 2 hours prior to cell harvesting [23,24].

The 8-Hydroxy-2-Deoxy Guanosine Assay. The 8-OHdG assay was performed as previously described [25]. In brief, blood cultures were set up by inoculating 0.5 mL of freshly drawn blood into 50 mL culture flasks containing 4.5 mL of PB-Max medium. Then, the cultures were incubated for 72 hours at 37 °C. Treatment was as described above. Cultures were then centrifuged at 1000 × g and 200 µL from each was used for the 8-OHdG assay. Competitive enzyme-linked-immunosorbent serologic assay (ELISA) for 8-OHdG were performed according to the manufacturer's protocol protocol (Abcam Inc., Cambridge, MA, USA). Plates were read at 405 nm using an Epoch Biotek microplate reader (BioTek, Winooski, VT, USA). Levels of 8-OHdG were calculated from the blotted standard curve.

Sister Chromatid Exchange Assay. A 25 µL of 0.01 g/L mL bromodeoxyridine (BrdU; Sigma-Aldrich, St. Louis, MO, USA), was added to the culture media prior to incubation and throughout the experiment [23,24]. All cultures were maintained in total darkness to minimize photolysis of BrdU [26-28]. The culture initiation and slide preparation were similar to that described for the chromosomal aberration assay. Air-dried slides were differentially stained by 10 µg/mL of Hoechst 32285 dye solution (Fluka, Munich, Germany) for 15 min., followed by rinsing in water and mounted in McILvian buffer (Fisher Scientific, Waltham, MA, USA) (pH 8.0). The slides were then irradiated with two UV lamps

(350 nm and 15 W each) at a distance of 7 cm for 35 min. at 40 °C [22]. Slides were then rinsed with distilled water, restained for 6-8 min. with 5.0% Giemsa in phosphate buffer (pH 6.8) and then air-dried [27,29,30]. Sister chromatid exchanges were scored using second division cells (M2, 50 cells per donor) that contained 42-46 chromosomes and high resolution (Nikon, Shinagawa, Japan) light microscopy [25,31]. Cells in M2 phase have two differentially stained chromatids, one lightly stained and one darkly stained, while M1 phase cells are uniformly stained (two chromatids are darkly stained), and M3 phase cells contain a mixture of lightly stained, darkly stained and differentially stained chromatids [26].

Cell Kinetics Analysis. The mitotic index was calculated by analyzing at least 1000 cells from each subject and scoring the cells that were in metaphase as previously described [26]. For the cell proliferation index, 100 metaphase cells from each donor were scored. The proliferation index was calculated using the following formula: $(1 \times M1 + 2 \times M2 + 3 \times M3) / 100$, where M1, M2 and M3 are the number of cells at the first, second and third metaphase, respectively [24,32]. Depending on the proliferation index, the average generation time was calculated as the number of hours for the cells in BrdU (Sigma-Aldrich), divided by the proliferation index [28].

Statistical Analyses. Statistical analysis was performed using Graphpad Prism statistical software version 4 (GraphPad Software, Inc., La Jolla, CA, USA). Data were expressed as mean \pm standard error

(SE). The comparisons of parameters were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Differences were regarded as significant at $p < 0.05$.

RESULTS

Figure 1 shows the level of 8-OHdG in all groups. Levels of 8-OHdG in drug-treated groups (VCR, VBL and VRL; each at concentrations of 0.01 and 0.1 $\mu\text{g}/\text{mL}$) were higher than those in the control group ($p < 0.05$). These results indicate that VCR, VBL and VRL at concentrations of 0.01 and 0.1 $\mu\text{g}/\text{mL}$ induce oxidative DNA damage. Figure 1 also shows that at both tested concentrations, the magnitude of the increase in 8-OHdG was higher in the VBL-treated group compared to the VCR and VRL groups (Figure 1).

Sister chromatid exchanges were observed in differentially stained M2 metaphase cells that have 42-48 chromosomes. There was an increase in SCEs frequency between control and drug-treated groups (VCR, VBL and VRL, each at concentrations of 0.01 and 0.1 $\mu\text{g}/\text{mL}$). However, this increase did not reach a significant level ($p > 0.05$) (Figure 2).

Previous studies showed that Vinca alkaloid drugs affect spindle fibers and causes cell arrest in the mitotic phase. As expected, the mitotic index was higher in the VCR-, VBL- and VRL-treated groups compared to the control group (Figure 3). In addition, in all treated groups, the effect was dose dependent.

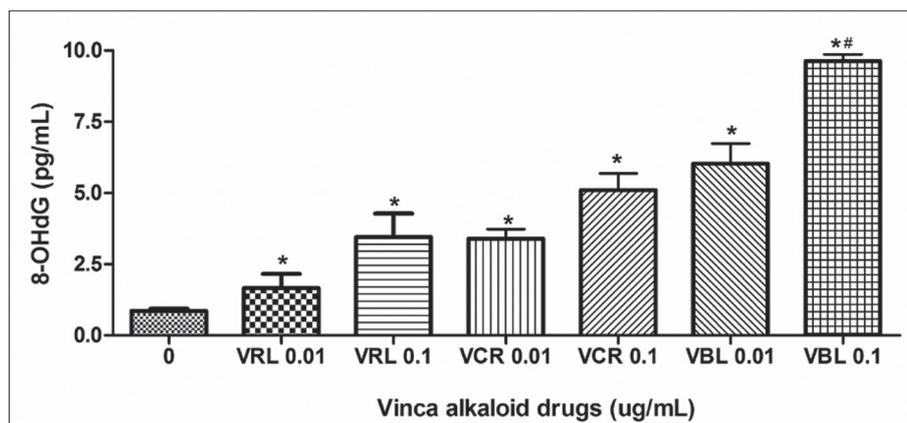


Figure 1. The level of 8-OHdG values in controls, VCR, VBL and VRL groups; each at concentrations of 0.01 and 0.1 $\mu\text{g}/\text{mL}$. The levels of 8-OHdG in all drug-treated groups at concentrations of 0.01 and 0.1 $\mu\text{g}/\text{mL}$ were higher than control group. The asterisks (*) indicate significant differences ($p < 0.05$) from the control group. The hash (#) indicates significant difference from all other groups.

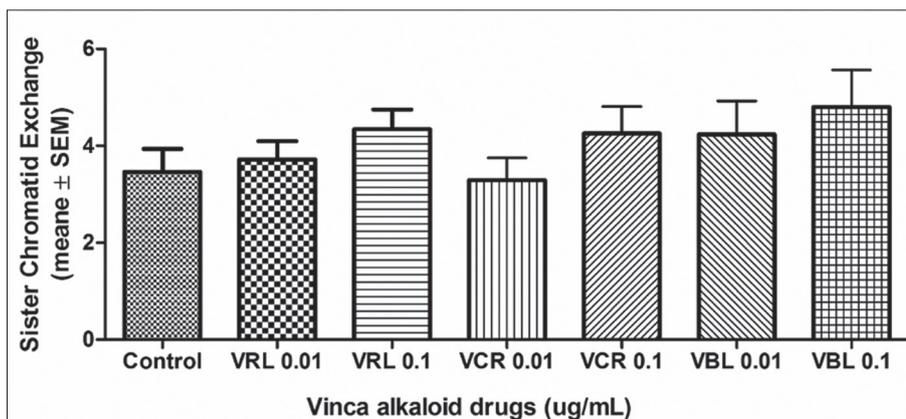


Figure 2. The average of SCEs/cell in controls, VCR, VBL and VRL groups; each at concentrations of 0.01 and 0.1 µg/mL in cultured blood cells. Increases were detected among the groups. However, it did not reach significant levels (control vs. VCR group: $p > 0.05$; control vs. VBL group: $p > 0.05$, and control vs. VRL group: $p > 0.05$).

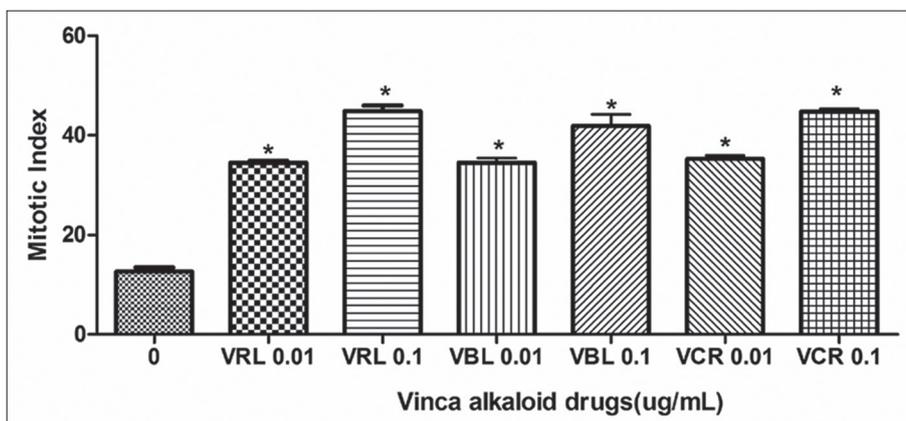


Figure 3. The mitotic index in cultured cells treated with VCR, VBL and VRL; each at concentrations of 0.01 and 0.1 µg/mL. The value of the mitotic index was significantly higher ($p < 0.05$) in lymphocytes treated with VCR, VBL and VRL; each at concentrations of 0.01 and 0.1 µg/mL.

Moreover, the magnitude of mitotic arrest was similar in VCR, VBL and VRL (Figure 3).

DISCUSSION

The current study indicated that VCR, VBL and VRL, at concentrations of 0.01 and 0.1 µg/mL, induced oxidative DNA damage as shown using 8-OHdG biomarker. The magnitude of the increase in 8-OHdG was higher in cells treated with VBL as compared to VCR and VRL.

The VCR, VBL and VRL have been reported to induce reactive oxygen and nitrogen species. For example, VRL has been shown to cause oxidative

injury to cultured human endothelial cells [33]. In addition, treatment of human bronchial epithelial cell with VCR was associated with oxidative DNA damage as measured using normal alkaline and formamidopyrimidine-DNA-glycosylase (FPG) modified comet assays [10,33,34]. It was recently reported that chronic treatment with antioxidant agents prevented che-motherapies, including VBL, induced oxidative damage and restores normal mitochondrial function in hepatic cells [35]. In this study, a significant increase in the level of 8-OHdG, which is a standard biomarker for oxidative DNA damage [36,37], was shown when cultured human lymphocytes were treated with VCR, VBL and VRL. This

variation could be due to the unique properties of these Vinca alkaloids (see Introduction). However, this point requires further investigation.

Current results show that at both tested doses, VBL induced increases in the levels of 8-OHdG that were larger in magnitude than those induced by both VCR and VRL. The exact mechanism for such amplified effect of VBL on the levels of 8-OHdG is unknown. However, previous studies have shown a differential effect for VBL on bone marrow cell as compared to fetal liver cells [17]. Additionally, VBL was shown to alter calcium homeostasis *via* mitochondrial membranes leading to substantiated cyto-toxicity as compared to VCR [38].

Results of this study showed no significant increase in SCEs frequency in the VCR-, VBL- and VRL-treated groups. Previous studies indicated the possible genotoxicity of VCR and VBL [12]. For example, VCR was shown to induce DNA misrepair, telomere end fusions, nuclear buds, and increased frequency of gene mutations, all of which points to the possible genotoxicity of VCR [10]. Vincristine was also shown to increase the number of micronucleated cells, indicating its possible genotoxicity [39,40]. All three agents, VCR, VBL and VRL, showed signs of genotoxicity in the *Drosophila* model [17]. On the other hand, VBL was recently shown to induce chromosomal aberrations and increase mitotic index *in vitro* in bone marrow cells, which was prevented by pretreatment with various doses of caffeine [41]. However, using the mouse lymphoma assay, VCR pretreatment for 3 hours did not show positive results for genotoxicity [42]. Additionally, increasing doses of VCR or VBL treatments were found to decrease the numbers of chromosomal aberrations along with an increase in number of micronucleated cells [11,43]. In another *in vivo* study in pregnant mice, a 2-week treatment with VCR did not induce an increase in SCEs in both maternal and fetal tissues, although it was capable of increasing the frequency of micronuclei [44]. Thus, it seems that Vinca alkaloids are able to induce certain types of DNA alterations such as oxidative and micronuclei but not others (*i.e.*, SCEs). This could be due to their mechanism of action that is associated with activation of apoptosis through interference with spindle fiber formation and mitochondrial function. Moreover, the aberrant variations in the genotoxicity results for VCR in *in vitro* cultured cell techniques and mammalian models could

be related to variation in models, doses and duration of drug treatment.

The current results showed increased mitotic index in cells treated with VCR, VBL and VRL, indicating an antimitotic activity of these compounds. This is consistent with previous results showing that VCR increased apoptotic cell numbers and ratios and decreased the nuclear division in a dose-dependent manner, thus, showing the cytotoxicity of VCR [10,40]. In fact, Vinca alkaloids including VCR, VBL and VRL, are known to exert their cytotoxicity *via* arresting mitosis and going into interphase [45,46], which is consistent with the current results. Based on the these results, we concluded that VCR, VBL and VRL induce oxidative DNA damage in whole blood lymphocytes.

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