

Original Article

Tempol protects human lymphocytes from genotoxicity induced by cisplatin

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Abstract: The use of cisplatin in treatments of human malignancies is limited by its side effects that include DNA damage and the subsequent risk of developing secondary cancer. In this study, we examined the possible protective effect of Tempol against DNA damage induced by cisplatin in human lymphocytes using chromosomal aberrations (CAs) and sister chromatid exchanges (SCEs) assays. Cisplatin induced significant elevation in the frequencies of CAs and SCEs in cultured human lymphocytes ($P < 0.01$). Treatment of lymphocytes with Tempol significantly lowered CAs and SCEs induced by cisplatin. Tempol alone did not affect spontaneous levels of SCEs and CAs observed in the control group ($P > 0.05$). In conclusion, Tempol protects human lymphocytes against genotoxicity induced by the anticancer drug cisplatin.

Keywords: Cisplatin, Tempol, chromosomal aberrations, sister chromatid exchanges, oxidative stress

Introduction

Cisplatin is a potent anticancer drug that is used for the treatment of many malignancies such as squamous cell carcinoma of the head and neck, testicular and ovarian cancer, uterine cervix carcinomas, and adrenocortical carcinoma [1, 2]. However, treatment with cisplatin is limited by its side effects which include nephrotoxicity, DNA damage and development of secondary tumors [3-6]. One of the suggested mechanisms by which cisplatin causes nephrotoxicity and tissue damage is the production of reactive oxygen species, depletion of oxidized form of glutathione and interference with activity of antioxidant enzymes [6-9]. This oxidative imbalance caused by cisplatin leads to accumulation of lipid and protein peroxidation, mitochondrial dysfunction and DNA damage [10-12]. Thus, finding a treatment that significantly limits such toxicity is essential for improvement of the therapeutic efficacy of cisplatin.

Tempol is a drug belonging to nitroxide compounds, which has been shown using animal models to be effective against oxidative tissue damage [13, 14]. For example, Tempol has been shown to protect mitochondria against oxidative damage by restoring oxidative bal-

ance inside cells [15]. In addition, oxidative damage caused by exposure to radiation is prevented by pretreatment with Tempol [16]. Moreover, Tempol protects animals against chronic diseases and deteriorations caused by aging-associated oxidative stress [17]. Recently, Tempol has been shown to prevent oxidative DNA damage caused by treatment of human cells with cadmium and chromium [18]. Similarly, Tempol has been shown to protect human lymphocytes from gamma-radiation induced genotoxicity [19]. Thus, Tempol might have the potential to be used as a protective drug against the oxidative stress associated with chemotherapeutic agents such as cisplatin. To test this hypothesis, the current study investigated the potential protective effect of Tempol against cisplatin induced DNA damage in human blood lymphocytes using sister-chromatid exchanges (SCEs) and chromosomal aberrations (CAs) assays.

Materials and methods

Subjects and experimental design

Five healthy male blood donors with age range of 20 to 27 years volunteered to give blood [20-23], which was obtained from them under ster-

ile conditions. Donors who were using alcohol, drugs, supplements, herbal medications, tobacco products were excluded from the study. Informed consent was obtained from each donor according to Institutional Review Board at Jordan University of Science and Technology. Blood samples were collected by venipuncture in heparinized tubes and were cultured within less than one hour of sampling [20, 24]. This study is a laboratory investigation that involved genotoxicity assessment of cisplatin and Tempol in human cultured lymphocytes obtained from healthy subjects using chromosomal aberrations (CAs) and sister chromatid exchanges (SCEs) assays.

Treatment with drugs

Cisplatin was obtained from Sigma-Aldrich (EC number 239-733-8, St. Louis, MO, USA). The drug was mixed with culture media and was added fresh to the cisplatin groups at a final concentration of 0.05 µg/mL and 0.8 µg/mL for SCEs and CAs experiments respectively [25]. These concentrations were within the IC50 range of cisplatin, which was reported by previous studies [26, 27]. Tempol was also obtained from Sigma-Aldrich (EC number 219-888-8St), and was dissolved in DMSO and was added fresh to Tempol groups at a final concentration of 10 µM [15, 21]. All drugs were added to blood cultures 12 hrs prior to lymphocytes harvesting. Experiments were performed on blood from each donor independently and were repeated from each donor twice.

Chromosomal aberrations assay

Blood cultures were initiated by adding 1 ml of fresh whole blood to 9 ml of complete karyotyping media (Gibco-Invitrogen, Paisley, UK). Cultures were incubated in the dark at 37°C for 72 hrs in a CO₂ incubator with appropriate humidity [28]. Two hrs prior to harvesting, Colcemid (0.1 µg/ml) was added to arrest the cells in metaphases. Cultured cells were harvested and fixed using methanol/acetic acid procedure as previously described [23, 29]. The cellular suspension was then dropped on pre-chilled microscope slides to obtain metaphase spreads. Slides were allowed to air dry and stained with 5% Giemsa stain (Sigma-Aldrich, St. Louis, MO, USA). CAs were evaluated in 100 well-spread metaphases containing 42-46 chromosomes per donor. Examination

was carried out using Nikon Clinical Microscope (Tokyo, Japan) at 1000x and the examiner was blind to the treatment. CAs were divided into gaps (including both chromatid gaps and chromosome gaps), breaks (including both chromatid breaks and chromosome breaks) and exchanges [30, 31].

Sister-chromatid exchanges assay

After initiation of blood cultures, a 5-bromodeoxyuridine (BrdUrd, Sigma-Aldrich) solution was added to a final concentration of 25 µg/ml. Lymphocyte harvesting and fixation were performed in a manner similar to the CAs assay described above. Slides were allowed to air dry and then differentially stained using a fluorescence-plus-Giemsa technique as previously described [24]. To score SCEs, 50 clearly differentiated second metaphases that contained between 42-46 chromosomes were examined for each donor.

Cell kinetics analysis

The mitotic index (MI) was calculated by analyzing 1,000 cells per treatment per donor and scoring the cells that were in metaphase. The cell proliferation index (PI) was calculated by scoring 100 metaphase cells per treatment as previously described [24].

Statistical analysis

Statistical tests were carried out using the GraphPad Prism software (version 5.0, LA Joelle, CA). ANOVA and Tukey post test were used for statistical evaluation between the groups. All values are represented as mean ± standard error of mean (SEM). Statistical differences were regarded as significant at $P < 0.05$.

Results

Chromosomal aberrations were observed in blood/human lymphocytes using plain Giemsa staining. Gaps, breaks and exchanges were included in the assessment. Treatment of cultures with cisplatin induced significant increases in chromosomal breaks, exchanges and gaps ($P < 0.05$, **Figures 1 and 2**). On the other hand, treatment of cultures with Tempol did not affect basal levels of chromosomal damage. However, treatment with Tempol significantly lowered chromosomal damage induced by cisplatin ($P < 0.05$, **Figures 1 and 2**). These results

Tempol effects on genotoxicity induced by cisplatin

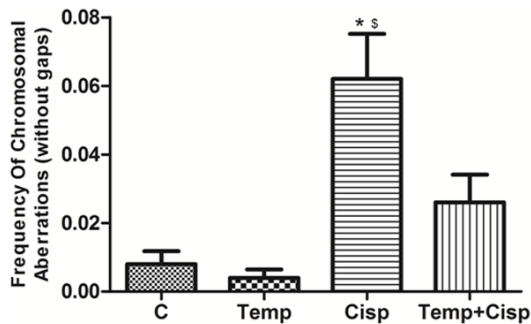


Figure 1. Levels of chromosomal aberrations without gaps in lymphocytes after treatment with cisplatin and Tempol. Chromosomal aberrations without gaps were examined in metaphase cells of cultured blood lymphocytes after treatment with Tempol and/or cisplatin. Data are expressed as mean \pm S.E.M. Cisplatin significantly increased chromosomal aberrations ($P < 0.01$). Tempol did not affect basal levels of chromosomal aberrations. However, Tempol significantly lowered chromosomal aberrations-induced by cisplatin ($P < 0.05$). *indicates significant difference from control group and Tempol group. \$indicates significant difference from temp + cisp group.

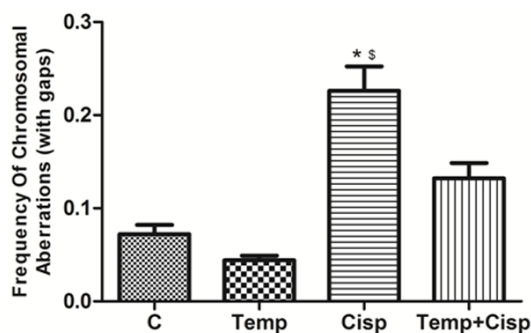


Figure 2. Levels of chromosomal aberrations with gaps in lymphocytes after treatment with cisplatin and Tempol. When gaps were included in the analysis of chromosomal aberrations easements induced by drugs, similar results to that without gaps were obtained: Cisplatin significantly increased chromosomal damage ($P < 0.01$). Tempol did not affect basal levels of chromosomal damage. However, Tempol significantly lowered chromosomal damage-induced by cisplatin ($P < 0.05$). Data are expressed as mean \pm S.E.M. *indicates significant difference from control group and Tempol group. \$indicates significant difference from temp + cisp group.

indicate a protective effect of Tempol against chromosomal damage induced by cisplatin.

To confirm the above result, the SCEs assay was used. Florescence-plus-Giemsa differentially stained M2 metaphase cells. Those that had 42-46 chromosomes were included in the

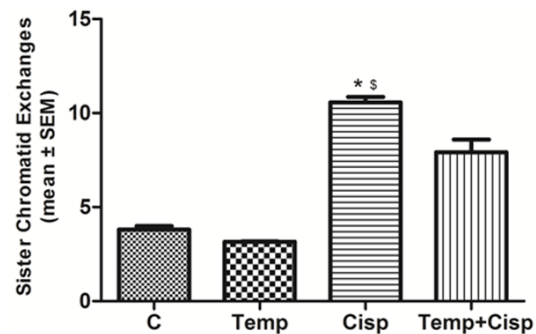


Figure 3. Frequencies of sister-chromatid exchanges in lymphocytes treated with drugs. Sister chromatid exchanges (SCEs) were examined in M2 cells of cultured blood lymphocytes. Data are expressed as mean \pm S.E.M. Cisplatin treatment significantly increased sister chromatid exchange frequency ($P < 0.01$), which was significantly reduced by Tempol ($P < 0.01$). Tempol by its self did not affect spontaneous SCEs level. *indicates significant difference from control group and Tempol group. \$indicates significant difference from temp + cisp group.

analysis. Similar to chromosomal aberrations, cisplatin induced significant elevation in the levels of SCEs whereas Tempol did not affect basal levels of exchanges (**Figure 3**). In addition, Tempol treatment significantly lowered (approximately 33% reduction) the levels of SCEs induced by cisplatin. However, the observed protective effect of Tempol against cisplatin-induced SCEs is partial as SCEs in "cisplatin + Tempol" group was significantly different from control group ($P < 0.05$, **Figure 3**).

SCEs data were further analyzed using the high frequency cells (HFCs) measure as previously described [29]. In this study, the threshold of HFCs that gives a percentile of 95 was found to be 8 SCEs/cell. The percentage of HFC in the control group was 4.4%. Approximately, 84.8%, 3.2% and 46.4% of M2 cells from cisplatin, Tempol and cisplatin + Tempol were HFCs respectively (**Table 1**). Thus, treatment with Tempol caused a significant decrease in the percentage of HFCs induced by cisplatin ($P < 0.01$). Thus, the HFCs analysis confirmed results obtained from those of the mean SCEs.

Mitotic index (MI) and proliferative index (PI) were used as an indicator reflecting the cytotoxicity of the examined drugs. No significant differences in MI between the different drugs or in combination were detected ($P > 0.05$, data not shown). Similarly, the PI was neither

Table 1. Distribution of SCEs per second-generation metaphase (M2) in human lymphocytes following treatment with cisplatin and/or Tempol

Treatment	Number of cells scored	Distribution of the number of SCEs per M2 cells (%)		
		0-3	4-7	≥ 8
Control	250	47.6	48	4.4
Tempol	250	63.2	33.6	3.2
Cisplatin	250	0.8*	14.4	84.8*
Tempol + Cisplatin	250	22.8	30.8	46.4

*indicates significant difference, $P < 0.05$.

affected by any treatment alone nor by the combined treatment ($P < 0.05$, data not shown).

Discussion

In this study, the protective effect of Tempol against cisplatin-induced genotoxicity was examined using CAs and SCEs assays. The results showed that the genotoxicity of cisplatin was significantly reduced by treatment with Tempol.

Cisplatin is widely used in chemotherapy for several human cancers. However, cisplatin is highly mutagenic, which raises concern about its potential to induce secondary cancers. The mutagenicity of cisplatin has been shown using different *in vivo* and *in vitro* systems. For example, using Swiss albino and Balb/c mice, cisplatin has been shown to induce chromosomal aberrations, micronuclei in bone marrow cells and DNA damage in blood lymphocytes and oxidative DNA damage in germ, brain and kidney cells [8, 32-37]. In rat bone-marrow, liver and heart cells, cisplatin also has been shown to induce DNA fragmentation, CAs and micronuclei [38, 39]. In addition, using the wing somatic mutation and recombination test, comet assay and wing-spot test, cisplatin has been shown to be mutagenic for *Drosophila* [40-42]. Finally, *In vitro* studies that used micronucleus test and comet assay showed DNA damage caused by cisplatin in PC12 cells [3], HepG2 human hepatoma cells [43], Ehrlich ascites tumour (EAT) cells of mice [44] and cultures of human Schwann cells [45].

The major suggested mechanism by which cisplatin exerts its genotoxic effect is by its ability to induce oxidative stress inside cells. Cisplatin has been shown to generate oxygen free radi-

cals, such as hydrogen peroxide, superoxide anions, hydroxyl radicals, and nitric oxide in kidney cells and blood of rat and mouse [34, 46, 47]. In addition, cisplatin causes modulation in the activity of catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase enzymes, [8], depletion in GSH in rats heart and liver cells [39] and elevation in lipid peroxidation, acetylcholinesterase activity and nitrite in the brain [36]. Finally, strong antioxidants have been shown to protect against genotoxicity of cisplatin. For

instance, genotoxicity of cisplatin in bone marrow and human lymphocytes has been shown to be reduced by organ selenium compound 2-(5-selenocyanato-pentyl)-benzo[de]isoquinoline 1,3-dione [34]. Cisplatin-induced chromosomal damage in bone marrow cells was prevented by *Cactus cladode* extract [8], resveratrol [37], *Hemidesmus indicus* root extract [48] and saffron [49]. DNA fragmentation and micronuclei formation by cisplatin in different mice organs were reduced by erythropoietin [39], Schisandrin B [36] *Crocus sativus* leaves extract [50] and Tannic acid [51]. Similarly, DNA damage in PC12 cells was prevented by bixin [3], glycyrrhizic acid [35] and curcumin [52]. The finding that Tempol reduced genotoxicity of cisplatin in cultured human lymphocytes agrees with previous literature and supports the role of oxidative stress in DNA damage induced by cisplatin.

The antioxidant activity of Tempol is well documented [15] and thus it is likely that Tempol protects against cisplatin induced genotoxicity through its neutralization of oxidative DNA damage. However, Tempol might protect against cisplatin-induced DNA damage by enhancing the rate of DNA repair. This was suggested by Ramachandran and Nair, who found an elevation in DNA repair index in cells exposed to Tempol and genotoxic agents as compared to cells exposed to genotoxic agents only [19]. This protection against genotoxicity of radiation did not modulate sensitivity of tumor cells to radiation. In accordance with this finding, Tempol significantly reduced the genotoxic effects of cadmium and chromium, by normalization of reactive oxygen species in cells [18]. More recently, Tempol has been shown to prevent genotoxicity in cultured human lymphocytes induced by vorinostat via modulation of

oxidative stress [21]. Thus, Tempol represents a promising drug with a potential use in cancer therapy to protect non-tumor cells from genotoxics of anticancer agents without affecting their potency.

In this study, the effect of Tempol against genotoxicity of cisplatin was examined on normal human lymphocytes. A major concern is that the antimutagenic effect of Tempol may protect cancer cells from the toxic effect of cisplatin. A recent study has shown that Tempol protects normal cells from genotoxicity induced by exposure to gamma radiation while maintaining radiation sensitivity of tumor cells [19]. In addition, treatments that lower oxidative stress enhance the effects of therapeutic agents against cancer cells [53, 54]. Exploring dose-response relationships and time courses of examined drugs, and mechanism by which Tempol protects against genotoxicity of chemical agents are granted in future studies.

In conclusion, Tempol has protective effect against cisplatin-induced genotoxicity and DNA damage in human lymphocytes.

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Disclosure of conflict of interest

Authors have no conflict of interest to report.

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