

Effects of cisplatin on the contractile function of thoracic aorta of Sprague-Dawley rats

YING JIANG^{1,2*}, SHIGANG SHAN^{1,3*}, TIEER GAN^{1*}, XIAOYUN ZHANG¹, XIANGHONG LU⁴, HU HU⁵, YIHUA WU¹, JIANZHONG SHENG⁵ and JUN YANG^{1,3}

¹The First Affiliated Hospital, State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, Zhejiang University, Hangzhou, Zhejiang 310003; ²Suzhou Biological Technology Co., Ltd., of Centre Testing International Corporation, Kunshan, Jiangsu 215300; ³Department of Toxicology, Hangzhou Normal University School of Public Health, Hangzhou, Zhejiang 310036; ⁴Department of Pharmacy, Lishui People's Hospital, Lishui, Zhejiang 323000; ⁵Department of Pathology and Pathophysiology, Zhejiang University School of Medicine, Hangzhou, Zhejiang 310058, P.R. China

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Abstract. DNA-damaging agents have been reported to be associated with cardiovascular complications, however, the underlying mechanisms remain to be clarified. In the present study, the possible vascular effects of cisplatin was assessed by measuring its effects on the contractile function of thoracic aortic rings dissected from Sprague-Dawley (SD) rats. Contraction of the aortic ring was induced by 60 mM KCl or 10^{-6} M phenylephrine (PE) in an *ex vivo* perfusion system. Cisplatin (200 μ M) counteracted KCl- and PE-induced contraction by 57.6 and 91.8%, respectively, in endothelium-intact aortic rings. Similar results were obtained in endothelium-denuded aortas. Electromicroscopy analysis revealed severe damage to blood vessel walls *in vivo* by cisplatin. In addition, cisplatin significantly inhibited adenosine triphosphate (ATP)-induced intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) increases in human umbilical vein endothelial cells (HUVECs). These results suggested that the DNA-damaging agent cisplatin can affect the contractile function of thoracic aortas. In addition, in accordance with its DNA-damaging properties, the cardiovascular toxicity of cisplatin may be the result of its direct cytotoxicity.

Introduction

DNA-damaging agents, or genotoxic agents, are those chemicals that can produce alterations in the genetic material of the host. Such agents can be further subdivided into direct- and indirect-acting agents. Direct-acting agents are intrinsically reactive and do not require metabolic activation by cellular enzymes to interact with DNA. By contrast, indirect-acting agents require metabolic activation by cellular enzymes to form the DNA-reactive metabolite. DNA-damaging agents exist widely in our natural and social environment, with examples including some of the chemotherapeutic agents and environmental pollutants (1).

Cisplatin, a direct-acting agent, is one of the most widely used chemotherapeutic agents in the treatment of a wide variety of malignancies (2). Although its biochemical mechanism of action has yet to be elucidated, cisplatin is believed to exert cytotoxic effects through the interaction and formation of adducts with DNA, which then leads to apoptosis and necrosis (3,4). Despite its clinical efficacy in treating malignancies, cisplatin-based chemotherapy regimens have been reported to be associated with vascular toxicity and serious vascular complications (e.g., myocardial infarction and stroke) (5-7). Such vascular toxicity has been manifested by increased von Willebrand factor plasma levels as well as an enhanced intima-media thickness of the carotid artery (8). In addition, cisplatin has the potential to induce ototoxicity and toxicity towards renal, peripheral sensory and autonomic nervous systems, potentially attributed to cisplatin-caused microvascular damage (9-11).

Ca^{2+} plays an important role in the regulation of vascular tone, which is generally relatively constant. Initiation of contraction in vascular smooth muscle is due to an increase in the free cytosolic Ca^{2+} concentration, which is caused by Ca^{2+} entry via adenosine triphosphate (ATP)-dependent ion channels (12). Aortic tension also plays an important role in regulating the entire cardiovascular system. Large arteries are involved in the regulation of hydrostatic capillary pres-

Correspondence to: Professor Jun Yang, The First Affiliated Hospital, State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, Zhejiang University, 79 Qingchun Road, Hangzhou, Zhejiang 310003
E-mail: junyanghzzju@163.com

Dr Jianzhong Sheng, Department of Pathology and Pathophysiology, Zhejiang University School of Medicine, 866 Yuhangtang Road, Hangzhou, Zhejiang 310058, P.R. China
E-mail: shengjz@zju.edu.cn

*Contributed equally

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sure (13,14) and blood pressure through changing pulsatile pressure (15). Arteries have a prominent cushioning effect in addition to their conducting function and this effect is associated with the viscoelastic properties of the arterial wall (16). In particular, the vasomotor reactions have been reported to be related to plasma volume and to help maintaining cardiovascular homeostasis (13). Although in previous studies it was reported that cisplatin is associated with a higher incidence of cardiovascular mortality and morbidity (17,18), the effect of cisplatin on vascular tone remains to be elucidated. Therefore, the aim of the present study was to evaluate the cardiovascular toxicity of cisplatin on the contractile function of rat thoracic aortas and to elucidate the possible underlying mechanisms.

Materials and methods

Reagents. Phenylephrine (PE), acetylcholine (ACh), cisplatin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Fluo-4/AM and ATP were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade and were purchased from Sinopharm Chemical Reagent (Shanghai, China). Cisplatin was dissolved in phosphate-buffered saline (PBS) for *in vitro* and *in vivo* experiments. Current guidelines for use of biohazardous materials in Zhejiang University were followed when using cisplatin.

Cell culture. Human umbilical vein endothelial cells (HUVECs) were grown in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (Gibco, Carlsbad, CA, USA) at 37°C under humidified air (5% CO₂).

Cytotoxicity assay. The toxicity of cisplatin was examined using the MTT assay. Briefly, cells were seeded into a 96-well culture plate at density of 1x10⁴ cells/well. After 24 h, the medium was discarded and replaced with fresh medium containing the indicated concentrations of chemicals. The cells were treated for 6 or 12 h and by the end of each time point, 20 µl of MTT (5 mg/ml in PBS) was added to each well and further incubated for 3 h. The solution was then discarded and 150 µl of isopropanol was added. Following agitation for 10 min to dissolve the formazan, the absorbance at 570 nm was read on a microtiter plate reader (BioTek, Winooski, VT, USA). Relative survival was represented as the absorbance of the treated sample/absorbance of the control group.

Animals and treatment of cisplatin. Male Sprague-Dawley (SD) rats weighing 230-260 g were obtained from the Laboratory Animal Center of Zhejiang University (Hangzhou, China). All the procedures were approved by the Ethics Committee for the Use of Experimental Animals in Zhejiang University. Rats were housed in an air-conditioned colony room at 20±2°C and diet and water were provided *ad libitum*. To detect the effect of cisplatin on thoracic aortas, the rats were injected intraperitoneally with cisplatin at a dose of 5 mg/kg body weight and an equivalent volume of saline was used as control (11).

Preparation of thoracic aortic rings. Rats were anaesthetized with 10% chloral hydrate (0.4 g/kg, intraperitoneally) and sacrificed by cervical dislocation. The chest was opened and the thoracic aorta was rapidly removed and placed in a 4°C

Krebs-Henseleit (K-H) solution [mM: NaCl, 120.0; KCl, 4.5; CaCl₂, 1.25; KH₂PO₄, 1.2; MgSO₄, 1.2; NaHCO₃, 25.0; and glucose, 10.0 (pH 7.4)]. Following removal of the superficial connective tissue, the aorta was cut into rings of 3-4 mm in length and subjected to contractile functional studies and electron microscopic measurements.

Electron microscopy. Three aortic rings cut from different segments of the thoracic aorta (superior, middle and inferior segment) were fixed with 2.5% glutaraldehyde at 4°C for >24 h and then washed twice in 0.1 M PBS at 4°C for 15 min. The tissue was incubated in 1% osmic acid at 4°C for 1 h and then rinsed twice in the same buffer for 15 min. After staining in 2% uranyl acetate (en bloc) at 4°C for 30 min, the tissues were processed by graded dehydration with different concentrations of ethanol (50, 70, 90 and 100%) and 100% acetone, respectively, each for 15 min. The dehydrated thoracic aortic rings were then agitated with a mixture of embedding medium (Epon 812) to acetone (1:1) for 1 h and then transferred into pure embedding medium (Epon 812) for another 1.5-2 h. Subsequently, the aortas were embedded with the same medium at 37, 45 and 60°C, each for 24, 24 and 48 h, respectively. Sections of ~120 nm were taken from the prepared rings using an ultramicrotome (Leica Microsystems, Milton Keynes, UK). The sections were stained with 4% uranyl acetate for 20 min and lead citrate for 5 min and then observed under transmission electron microscopy (Tecnai 10, 80 kV). The number of smooth muscle cells was counted depending on the image (x970) captured from electron microscopy.

Measurement of contractile function of thoracic aortic rings. Fresh isolated rings were mounted in 5.0 ml organ baths containing the K-H solution. The bath solution was maintained at 37°C and bubbled continuously with a gas mixture of 95% O₂ and 5% CO₂. Aortic rings were equilibrated for 60 min at resting tensions of 2 g. Isometric tension was measured with force isometric transducers connected to a data acquisition system (MedLab; Nanjing Medease Co., Ltd., Nanjing, China). In some aortic rings, the endothelium was mechanically removed by gentle rubbing with moistened cotton. The removal of endothelial cells was confirmed by the loss of ACh-induced relaxation (19-21). After 6 h pre-incubation with cisplatin (200 µM), contraction of the endothelium-intact and endothelium-denuded aortic rings was induced by KCl (60 mM) or PE (10⁻⁶ M). The vascular response was expressed as a percentage of the 'plateau' contraction evoked by KCl or PE (22).

Measurement of intracellular Ca²⁺ concentration ([Ca²⁺]_i). HUVECs grown on coverslips were treated with cisplatin (200 µM) for 6 h. The cells were loaded with the Ca²⁺ sensitive dye Fluo-4/AM (1 nM) for 30 min at 37°C in serum-free RPMI-1640 medium. After loading, the cells were washed three times with serum-free RPMI-1640 medium. The loaded cells were maintained at 37°C for 15 min before measurement of [Ca²⁺]_i in order to allow the Fluo-4/AM in the cytosol to deesterify. The cells were subsequently placed on the stage of a Nikon inverted microscope equipped with a SFX-1 microfluorimeter (Solamere Technology Group, Salt Lake City, UT, USA). During the experiments, the cells were superfused in a 0.3-ml bath chamber at 35°C under a constant flow

Table I. The relative baseline tension of aortic rings treated with cisplatin.

Thoracic aortic rings	Control (n)	Cisplatin (n)			
		10 μ M	100 μ M	200 μ M	500 μ M
+E	0.018 \pm 0.036 (6)	0.033 \pm 0.045 (5)	0.073 \pm 0.054 (6)	0.091 \pm 0.063 (6)	0.059 \pm 0.034 (5)
-E	0.033 \pm 0.052 (9)	0.037 \pm 0.050 (5)	0.056 \pm 0.058 (6)	0.021 \pm 0.045 (7)	0.063 \pm 0.069 (5)

Thoracic aortic rings (+E, endothelium-intact thoracic aorta; -E, endothelium-denuded thoracic aorta) were incubated in various concentrations of cisplatin (μ M) for 6 h. Relative tensions of aortic rings were evaluated by *ex vivo* perfusion. Data are expressed as mean \pm SEM.

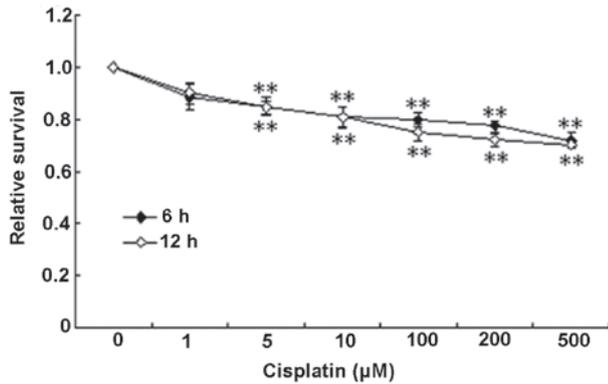


Figure 1. The cytotoxicity of cisplatin on human umbilical vein endothelial cells (HUVECs). HUVECs were treated with various doses of cisplatin for 6 or 12 h and cell viability was measured by the MTT test. Data are expressed as mean \pm SEM. **P<0.01 compared to the control.

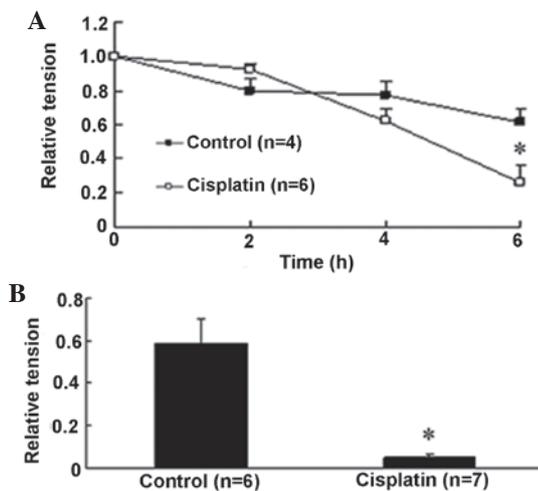


Figure 2. Effect of cisplatin on the relative tension of endothelium-intact thoracic aorta. (A) Cisplatin decreased the relative tension of endothelium-intact rat thoracic aortic rings precontracted with KCl at the resting tension of 2 g in an *ex vivo* perfusion system in a time-dependent manner. (B) Cisplatin inhibited the contraction induced by phenylephrine in endothelium-intact rat thoracic aortic rings at 6 h. Data are expressed as mean \pm SEM. *P<0.05 versus control endothelium-intact aorta rings.

(1 ml/min) of HEPES-buffered saline (135 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose and 10 mM HEPES; pH 7.4) (23). To examine the action of cisplatin on ATP-induced increased [Ca²⁺]_i, the cells were stimulated with ATP (10 μ M) for 1 min. ATP was then washed away twice

by HEPES-buffered saline. Fluo-4 fluorescence was excited at 470 nm. Emitted Fluo-4 fluorescence was measured at wavelengths of 520 nm. Changes in [Ca²⁺]_i were reported as the Fluo-4/AM ratio $\Delta F/\Delta F_0$, where ΔF is the difference between baseline fluorescence intensity and maximal fluorescence intensity after ATP stimulation, while ΔF_0 is the difference between baseline fluorescence intensity and the minimal fluorescence intensity prior to ATP stimulation.

Statistical analysis. Data were expressed as mean \pm standard error of the mean (SEM). Statistical analysis was carried out using Student's paired t-test or one-way analysis of variance followed by Dunnett's t test. Differences were considered statistically significant at P<0.05.

Results

Effects of cisplatin on the contractile function of thoracic aortas of SD rats. First the cytotoxicity of cisplatin was examined by MTT using HUVECs to determine the appropriate dose for subsequent experimentation. As shown in Fig. 1, cisplatin at doses ranging 5-500 μ M induced cytotoxicity at 6 and 12 h in a dose-dependent manner, with maximal decreases in cell survival (20-30%) observed at the higher concentrations.

Using the *ex vivo* perfusion system, the effects of cisplatin on the contraction of aortic rings were examined. At concentrations ranging 10-500 μ M, cisplatin itself had no direct effect on the baseline tension of endothelium-intact aortic rings (Table I). Combined with the cytotoxicity data, 200 μ M was selected as the concentration for subsequent experimentation. KCl (60 mM) induced significant contraction with the relative tension as 1 at time 0 and gradually decreased to 0.61 \pm 0.08 after 6 h. It was found that cisplatin decreased the contraction tension provoked by KCl in a time-dependent manner, with a maximum inhibition of 57.6% (0.26 \pm 0.10, P<0.05 versus 0.61 \pm 0.08) at 6 h (Fig. 2A). PE (10⁻⁶ M) increased the contraction tension to a similar level as KCl (0.59 \pm 0.12) at 6 h and it was almost abolished by cisplatin, as shown in Fig. 2B, in which cisplatin reduced PE-induced contraction tension by 91.8% (0.049 \pm 0.01, P<0.05 versus 0.59 \pm 0.12).

Effects of cisplatin on the contractile function of endothelium-denuded thoracic aortas of SD rats. Endothelium is known to play an important role in regulating the vascular tone by releasing factors involved in relaxation and contraction (24). Therefore, we also assessed the effects of cisplatin on endothelium-denuded aortic rings. In the aortic rings of

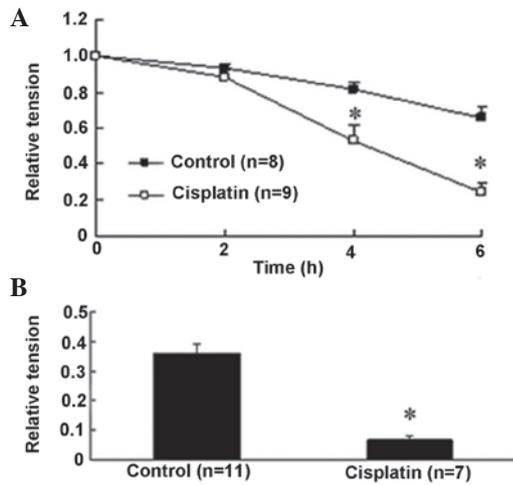


Figure 3. Effect of cisplatin on the relative tension of endothelium-denuded thoracic aorta. (A) Cisplatin decreased the relative tension of endothelium-denuded rat thoracic aortic rings precontracted with KCl at the resting tension of 2 g in an *ex vivo* perfusion system in a time-dependent manner. (B) Cisplatin inhibited the contraction induced by phenylephrine in endothelium-denuded rat thoracic aortic rings at 6 h. Data are expressed as mean \pm SEM. * $P < 0.05$ versus control endothelium-denuded aorta rings.

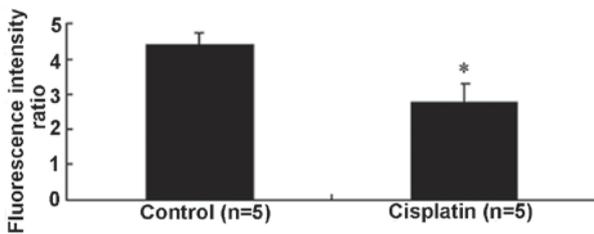


Figure 4. Effects of cisplatin on adenosine triphosphate-induced changes of intracellular Ca^{2+} concentration in human umbilical vein endothelial cells. Data are expressed as mean \pm SEM. * $P < 0.05$ versus control.

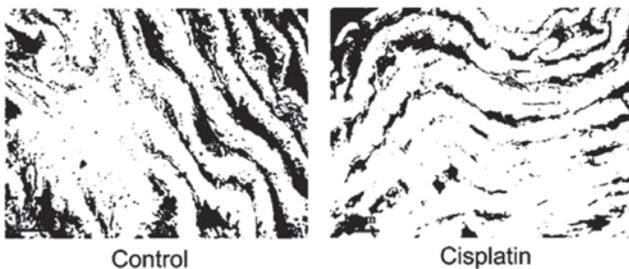


Figure 5. Cisplatin induced damages in the smooth muscle layer in Sprague-Dawley rats. Representative images of thoracic aorta segments (superior segment of aortas) from control and cisplatin-treated rats are shown.

endothelium denudation, KCl and PE also increased vascular tension to 0.65 ± 0.07 and 0.36 ± 0.03 at 6 h, respectively (Fig. 3). Similar to the endothelium-intact situation, cisplatin decreased KCl-induced contraction tension by 63.0% (0.24 ± 0.05 , $P < 0.05$ versus 0.65 ± 0.07) and PE-induced contraction tension by 81.3% (0.067 ± 0.01 , $P < 0.05$ versus 0.36 ± 0.03).

Effect of cisplatin on $[Ca^{2+}]_i$ in HUVECs. As stated before, since $[Ca^{2+}]_i$ is also an important factor in regulating vascular tone, the effect of cisplatin on $[Ca^{2+}]_i$ was examined in

HUVECs. As shown in Fig. 4, cisplatin ($200 \mu\text{M}$) significantly decreased the $[Ca^{2+}]_i$ in HUVECs increased by ATP (2.79 ± 0.58 versus 4.42 ± 0.33).

Effects of cisplatin on aortic vessel wall components in vivo. To examine the effects of cisplatin on the aortic walls, aortas from the control- and cisplatin-treated rats were isolated and processed for electron microscopic observation. As shown in Fig. 5, cisplatin caused severe damage to the smooth muscle layer. Compared to the control, the boundary of smooth muscle layers was less clearly defined and almost touching each other in the aortas from cisplatin-treated rats. Cisplatin also caused significant loss of smooth muscle cells, for example, the average smooth muscle cell count was decreased by 34.7% (from $26,383 \pm 2,561$ to $17,217 \pm 1,452 \text{ mm}^{-2}$ with cisplatin treatment, $P < 0.05$).

Discussion

In the present study, by measuring the contractile function of thoracic aortas in an *ex vivo* perfusion system, we evaluated the vascular effects of the DNA-damaging agent cisplatin. It has been reported that there is a high incidence of cardiovascular events in survivors of cisplatin-based chemotherapy cancer patients (25), either in acute or long-term chemotherapy (26,27). Cisplatin was reported to lead to aortic occlusion in some cases and this impairment was usually irreversible (28,29). In one case, a 36-year-old man who was treated with bleomycin-etoposide-cisplatin therapy for testicular cancer received chronic coronary artery dissection (30). A long-term follow-up in a large cohort of testicular cancer survivors showed that compared with healthy controls, cisplatin-treated patients had, at follow-up, increased systolic blood pressure, increased diastolic blood pressure and a higher prevalence of hypertension (18). In that cohort, patients in the high-dose cisplatin-treated group had increased odds for having intermediate/high risk in cardiovascular mortality, compared with the surgery group (OR=3.4, 95% CI: 1.3-8.7) (17). All of the above case reports led to the hypothesis that cisplatin may cause deleterious effects on the cardiovascular system. Thus, we undertook a first step to investigate whether cisplatin has such an effect and in particular, we focused on the contractile function of aortic rings as an indicator.

First, we demonstrated that the baseline tensions of thoracic aortas were not altered by cisplatin treatment, which means that cisplatin had no direct deleterious effect on the contractile function of aortas. However, cisplatin inhibited KCl- or PE-induced contraction in the endothelium-intact and endothelium-denuded aortas, suggesting an endothelium-independent mechanism (Figs. 2 and 3). To investigate the reason for cisplatin decreasing the tension of aortic rings, we examined the structure of the aortic walls. As shown in Fig. 5, cisplatin severely damaged the smooth muscle layer, and significantly decreased smooth muscle cells in the thoracic aortic wall. The fundamental structural and functional unit of the aortic wall is the medial lamellar unit. The media layer comprises elastic membrane layers between which are the smooth muscle layer and a small amount of collagen and elastic fibers. The integrity of the vascular smooth muscle layer is crucial in the maintenance of normal vascular morphology and tone. Changes

in arterial wall composition and function underlie all forms of vascular disease (31). Therefore, the finding that cisplatin damaged the arterial wall may partially explain its inhibitory effect on the contraction of aortic rings.

In addition, we have also shown that cisplatin inhibited ATP-induced Ca²⁺ influx in HUVECs (Fig. 4). However, whether cisplatin similarly affects Ca²⁺ in aortic tissues remains to be determined.

The effects of another environmentally prevalent DNA damaging agent, benzo(a)pyrene [B(a)P] on the contractile function of thoracic aortas have also been investigated. Unlike cisplatin, B(a)P is an indirect-acting agent and has to be metabolically processed by aryl hydrocarbon receptor-induced enzymes, such as cytochrome P4501A1 (CYP1A1) and epoxide hydrolase, to form carcinogenic active form B(a)P-diol epoxides (32). Of note, B(a)P only affected the contractile function of thoracic aortas following long-term *in vivo* exposure (4 weeks) (data not shown).

In conclusion, in the present study, we have shown that the DNA-damaging agent cisplatin: i) decreased the contractile function of thoracic aortas; and ii) caused direct vessel wall damage and cytotoxicity towards smooth muscle cells. Therefore, it seems that DNA-damaging agents may also have cardiovascular toxicity and thus, the potential cardiovascular complications associated with DNA-damaging agents should be investigated in future studies.

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