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Modulatory Effect of *Zingiber Officinale* Oil on Oxidative Stress and DNA Damage in Etoposide-Treated Rats

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Abstract

Etoposide (ETZ) is a standard drug used in treatment of several malignancies. However, it is associated with increased risk of spleen damage and secondary acute myeloid leukaemia. Ginger (*Zingiber officinale*) is an ancient safe remedy used in management of minor health issues due to being rich in active constituents. Therefore, we hypothesize that ginger oil can ameliorate ETZ-induced oxidative stress and DNA damage in spleen tissues and leukocytes. To achieve our aim, ginger oil (75&150 mg/kg b.w.) was administered three times to male albino rats an hour prior to ETZ oral administration (1mg/kg b.w.) for 3 weeks. Results showed that ETZ induced marked biochemical alterations in the oxidative status of spleen tissues. The elevated lipid peroxidation and nitric oxide levels were statistically countered. However, the glutathione, superoxide dismutase and catalase enzymes were reduced. Moreover, the DNA single strand breaks as evaluated by comet assay in blood leucocytes also showed significant changes. The administration of ginger oil caused significant improvement of oxidative stress status and DNA damage. In conclusion, our results suggest that ginger oil could be used as a herbal remedy for reducing ETZ-induced adverse effects in male albino rats.

Keywords: Etoposide, Ginger oil, DNA damage, Comet assay, Oxidative stress, Spleen.

INTRODUCTION

Etoposide (ETZ) derives from podophyllotoxin, a toxin present in the American Mayapple. It belongs to a class of anticancer drugs called plant alkaloids. It interferes with the action of topoisomerase I and II enzymes. Topoisomerase enzymes control the manipulation of the structure of DNA necessary for replication [1]. Etoposide is used in the treatment of kposi's sarcoma, testicular cancer, lymphoma, nonlymphocytic leukemia, lung cancer, and glioblastoma multiforme [2]. Bone marrow suppression, anemia, leukopenia, low blood pressure, thrompocytopenia, abdominal pain, constipation, dysphagia, fatigue, malaise, optic neuritis, pulmonary fibrosis, cardiac toxicity, vomiting, nausea, fever, acute myeloid leukemia, hair loss and bleeding are the most common side effects that result from the treatment with ETZ [3]. Etoposide may be hazardous during pregnancy and can cause fetal harm and disorder in the functions of kidneys, liver and spleen [4].

However, ETZ is ananticancer drug inducing breaks in cellular DNA strand by inhibiting topoisomerase II (topoII) religation of cleaved DNA molecules. Although DNA cleavage by topoisomerase II always produces topoisomerase II-linked DNA double-strand breaks (DSBs). In addition, the action of ETZ leads to single-strand breaks (SSBs), since religation of the two strands are independently inhibited by ETZ [5]. Furthermore, ETZ increased lipid peroxidation and nitric oxide (NO) levels. It reduced glutathione (GSH) levels and catalase (CAT) activities in the kidney [6]. ETZ induced chromosomal aberrations and liver tissues DNA damage in rats [7].

Medicinal plants are well known traditionally to be used in the treatment of various diseases including anticancer potential [8-10] and antioxidant properties [11]. The phytomedicines exert their effect by various mechanisms such as antioxidant and cytoprotective actions [12].

Ginger (*Zingiber officinale* Roscoe, Family: *Zingiberaceae*) has been used in the treatment of headaches, cold, diarrhea, gastrointestinal disturbances, nausea, asthma and rheumatic complaints, arthritis and

muscular discomfort [13]. Furthermore, it has been used as anti-inflammatory, cardio-protective, anticancer agent. However, the antioxidant, anti inflammatory and antimutagenic properties of ginger were reported [14] and attributed to its active components that induce apoptosis in skin, ovarian, colon, breast, cervical, oral, renal, prostate, gastric, pancreatic, liver and brain cancers. Its extracts may enhance the immune action by regulating the splenocyte proliferation and cytokine production capacity by activated macrophages in mice [15]. Ginger has a protective effect on spleen marginal zone and germinal centre of diabetic guinea pigs [16]. It ameliorated the genotoxicity and cytotoxicity induced by carbimazole in albino rats [17], decreased chromosomal aberrations [18] and protected DNA damage expressed as a comet ratio induced by Benzopyrene in human peripheral blood lymphocytes [19].

Based on the mentioned data, this study aimed to evaluate the putative protective effect of GO in ETZ-intoxicated rats.

MATERIAL AND METHODS

Materials

Etoposide (etopophos), $C_{29}H_{32}O_{13}$, (4'Demethylepipodophyllotoxin9-[4,6-O-(R)-ethylidene-β-D glucopyranosi- de], 4'-(dihydrogen phosphate) was purchased from a local pharmacy.

Ginger oil (*Zingiber officinale* Roscoe) was obtained from Andalos Import and Export Company (99.9%), Egypt.

Experimental Animals

Housing and Grouping of the Animal

Thirty adult male albino rats (*Rattus norvgicus*) weighting about 200±10 g were used for the present study. Initially, rats were obtained from the Serum and Antigen Laboratories, Helwan, Egypt. Animals were acclimatized for a period of two weeks in the animal house.The animal handleing and care followed the Institutional Animal Ethical Committee (IAEC) guidelines of Zoology Department, Faculty of Science, Menoufia University (2-17-Sc-MNF). The animals were housed in clean cages and maintained under controlled conditions of temperature (25 ±1.5ºC), light (12 light: 12 dark hours) and good ventilation. They were fed a standard rodient diet (Al Nasr Co., Egypt) and water *ad libitum*. Rats were equally divided into six groups (5 rats/group). The first group served as control (untreated) group. Group 2, received ETZ orally (1mg/kg b.wt.); Group 3, received GO (75mg/kg b.wt.); Group 4, received GO (150mg/kg b.wt.); Group 5, received ETZ+GO (75mg/kg b.wt.); Group 6, received ETZ+GO (150mg/kg b.wt.). Animals received all treatments orally by stomach tube three times weekly for 21 days. The applied doses in this study were according to hassab El Nabi *et al.*, [7].

METHODS

Investigations of anti-genotoxicity

Collection and isolation of blood leucocytes

Blood was collected from unanesthetized rats [20]. In compliance with the laboratory animal care guidelines, about 2 ml of blood per rat was collected in K_2EDTA tubes (KEMICO vacutainer, Egypt), by a submandibular bleeding method. For hemostasis, a gentle pressure was applied to the punctured area.

Alkaline single cell gel electrophoresis (Comet assay)

This technique is widely used for detection of DNA single-stranded breaks and alkali-labile sites [21]. Cells were embedded in 0.7% low melting agarose between two layers of 0.5% ultra-pure agarose on a clean microscopic slide. The cells then were lysed by detergents and high salt solution at pH 13 then allowed to denature the DNA molecuoles in the running buffer for 15 min. Samples were electrophoresed for damage display showing the increased migration of DNA towards the anode. All chemicals were obtained from Sigma-Aldrich, Germany. Five hundred nuclei were counted and classified according to the length of DNA tail using a fluorescent microscope (Olympus BX 41, Japan).

Total Genomic DNA Fragmentation Assay

In order to assess the genotoxic effect of ETZ on spleen tissues, double-strand breaks of DNA was evaluated in treated animals and controls according to "salting out extraction method" of Aljanabi and Martinez [22] with some technical modification according to Hassab El-Nabi and Elhassaneen [23]. About 20mg of spleen tissues were lysed in 500 µl of lysing buffer (50 mM NaCl, 1mM Na_2 EDTA, 0.5% SDS, pH 8.3). Proteins and cellular contents were precipitated by 5M NaCl. Nucleic acids were precipitated by cold isopropanol. The pellets were washed twice with 70% ethanol. The pellets were re-suspended in appropriate volume of TE buffer (10mM Tris, 1 mM EDTA, pH 8). The re-suspended pellets were incubated for 30-60 min., with loading buffer supplemented with RNase. Samples were directly stained with ethidium bromide [24], and then loaded directly into the 1.5% agarose gel. Apoptotic bands of DNA fragmentation appeared and were located at 180 bp and its multiples 360, 540 and 720bp against 100bp DNA ladder (Thermo Scientific™ O'gene ruler™). The intensity of released DNA fragments was measured by image J software, as a mean of optical density values.

Oxidative stress investigations

Preparation of spleen tissue homogenate and protein determination

Spleen tissues were homogenized in 0.1 M phosphate buffer at pH 7.4 to prepare 10% homogenate then centrifuged for 15 min at 4°C at 4,000 rpm to obtain the supernatant.The protein concentration in the supernatants was determined according to the method of Lowry *et al.*, [25].

Oxidative stress parameters

Lipid peroxidation was determined by colorimetric measurment of the malondialdehyde (MDA) concentration at 534 nm according to Ohkawa *et al*., [26] and the MDA values were expressed as nmol of MDA/gm protien. Abnormal accumulation of the stable product of nitric oxide (NO) nitrite was determined according to Granger [27]. The reduced glutathione (GSH) levels were determined colorimetrically at 412 nm as described by Beutler *et al*., [28]. Catalase (CAT) activity following the method of Fossati [29]. And superoxide dismutase (SOD) activity was determined spectrophotometrically at 560 nm according to Nishikimi *et al*., [30].

Statistical Analysis

In the present study, data were represented as means± standard deviation (SD). Comparisons were performed between the untreated and treated groups. All data were statistically analyzed using Statistical Program of Social Science (SPSS) [31].The $P \leq 0.05$ was considered significant.

RESULTS

General Health

Etoposide treated rats showed no mortality during the entire period of treatment. No irritation at the ETZ injection sites was observed.

Assessment of Genotoxicity

DNA Single-Stranded Breaks and Alkali-Labile Sites

Single cell gel electrophoresis of leukocytes for treated animals and controls were done after 72 hours of treatments (Figure-1). The results revealed the significant ($P< 0.05$) DNA damage in the etoposide-treated group (40.0 ± 2.68) with respect to control (5.0 ± 1.78). However, the treatment with 150 mg/kg b.wt. of GO exerted DNA damage of a percentage of 21.0 ± 3.22. In case of combination, it failed to protect DNA from the damage of ETZ and the record was 59.3 ± 1.78. Moreover, the dose of GO (75mg/kg b.wt.) showed a protective effect amounting to about 26% (Figure-2).

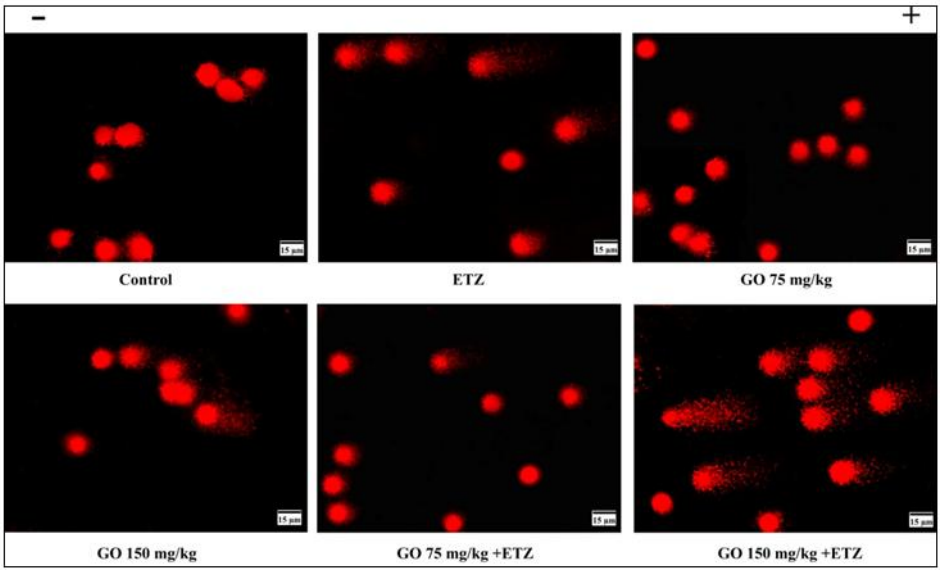

Fig-1: Representative photomicrograph of single cell gel electrophoresis (Comet assay) showing the effect of different treatments on single strand breaks of rat's peripheral blood leukocytes. The normal DNA spots (no migration) and damaged DNA spot (migration towards the anode). ETZ, Etoposide (1mg/kg b.wt.); (n=5).

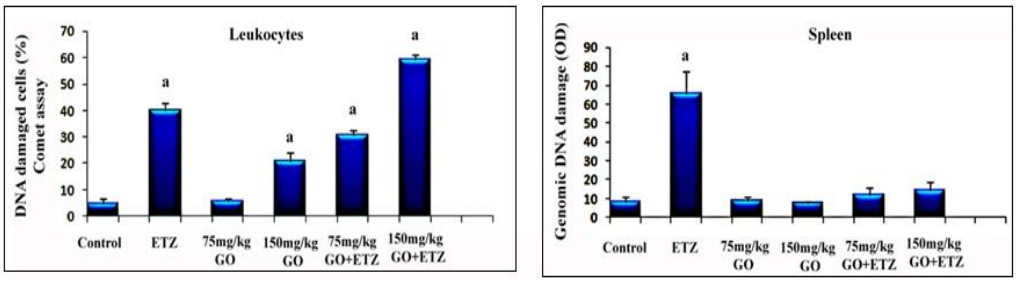

Fig-2: The protective effect of ginger oil against etoposide-induced DNA damage. The protective effect of ginger oil (GO) treatments (75 &150mg/kg b.wt.) on total released genomic DNA fragmentation in spleen tissues of ETZ-treated rats (Right panel, 21th day). However, the ameliorative effect of GO dosing on single strand breaks (Comet assay) of peripheral blood leukocytes in ETZ-treated rats (Left panel, 72h). Data were represented as means of three independent experiments (n=5) and bars for standard deviation.ETZ, etoposide (1mg/kg b.wt.); GO, Ginger oil; a, statistically significant ($P \leq 0.05$) with respect to control.

Genomic DNA fragmentation in spleen tissues

Etoposide induced severe damage in genomic DNA of spleen tissues as apparent in the form of DNA laddering fragmentation (apoptosis) after 21 days of treatment.The optical density value of fragmented DNA was 65.8±3.30 that showed significant increase ($P \leq 0.05$) with respect to the control group with a value of 8.6±0.53. However, the treatment with 75& 150 mg/kg b.wt. of GO revealed normal appearance of intact DNA (Figure-3). Furthermore, the combined treatment of GO (75& 150 mg/kg b.wt.) with ETZ showed a protective effect reaching 90 and 80%, respectively, against ETZ damaging effect (Figure-2).

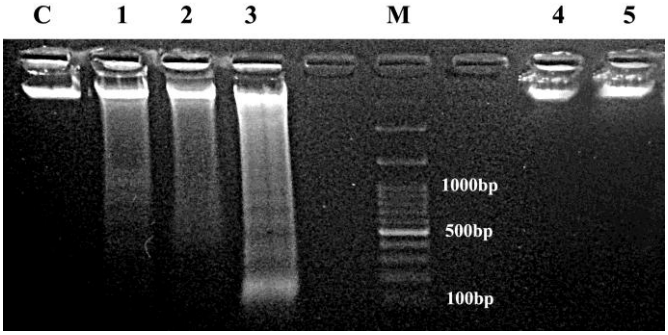

Fig-3: Representative digital photograph of genomic DNA electrophoresis of rat's spleen tissues on 1.8% agarose gel showing the protective effect of ginger oil against etoposide-induced damage. Where C: untreated group; 1: combination of ETZ+G.O (75mg/kg b.wt.); 2: ETZ+G.O (150mg/kg b.wt.); 3: ETZ (1mg/kg b.wt.); 4: G.O (75mg/kg b.wt.); 5:G.O (150mg/kg b.wt.) and M: DNA ladder.

Oxidative status in spleen tissues

The effect of GO on lipid peroxidation levels

Etoposide induced significant elevation of MDA levels when compared to the untreated group. Treatment with GO ameliorated the effect of ETZ; especially at the lower dose. The protective effect of GO (75 &150mg/kg b.wt.) against etoposide reached about 100 and 70%, respectively (Figure-4).

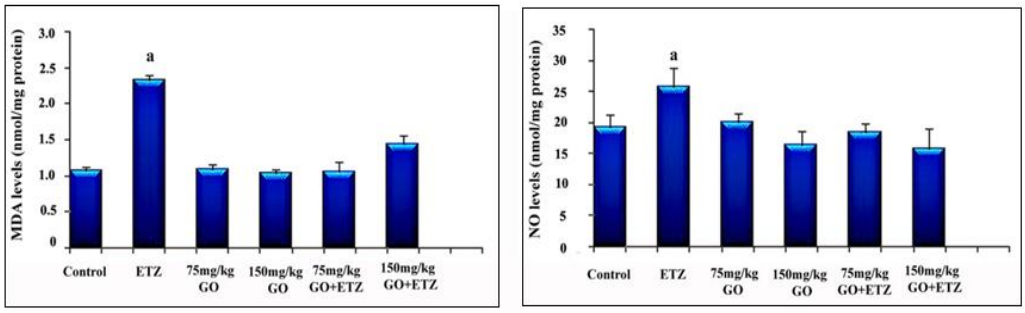

Fig-4: The protective effect of ginger oil (75 &150mg/kg b.wt.) against etoposide-induced oxidative stress. The lipid peroxidation as measured as malondialdhyde (MDA), (Left panel) in spleen tissues of ETZ-treated rats and nitric oxide levels (NO), (Right panel). Data were represented as means of three independent experiments (n=5) and bars for standard deviation. ETZ, etoposide (1mg/kg b.wt.); GO, Ginger oil; a, statistically significant ($P \leq 0.05$) with respect to control

The effect of GO on nitric oxide levels

Etoposide induced significant increase in NO levels relative to the control group. Treatment with both doses of GO ameliorated the effect of ETZ and decreased the NO levels. The protective effect of GO (75 &150mg/kg b.wt.) against etoposide reached about 100% (Figure-4).

The effect of GO on superoxide dismutase activities.

Etoposide induced marked decrease in SOD activity versus the control group. Treatment with both doses of GO ameliorated the effect of ETZ and elevated the SOD levels. The protective effect of GO (75 &150mg/kg b.wt.) against etoposide reached about 97 and 80%, respectively. However, the lower dose of GO exerted better effect on the SOD levels (Figure-5).

The effect of GO on catalase activities

Etoposide induced marked decrease in CAT activity when compared to the control group. Treatment with both doses of GO ameliorated the effect of ETZ and elevated the CAT activities. The protective effect of GO (75 &150mg/kg b.wt.) against etoposide reached about 71 and 15%, respectively. However, the lower dose of GO exerted better effect on the CAT activities (Figure-5).

The effect of GO on glutathione levels

Etoposide induced significant ($P < 0.05$) decrease in GSH level in comparison with the control group. Treatment with both doses of GO ameliorated the effect of ETZ and elevated the GSH levels. The protective effect of GO (75 &150mg/kg b.wt.) against etoposide reached about 77 and 17%, respectively. However, the lower dose of GO exerted better affects towards the GSH levels (Figure-5).

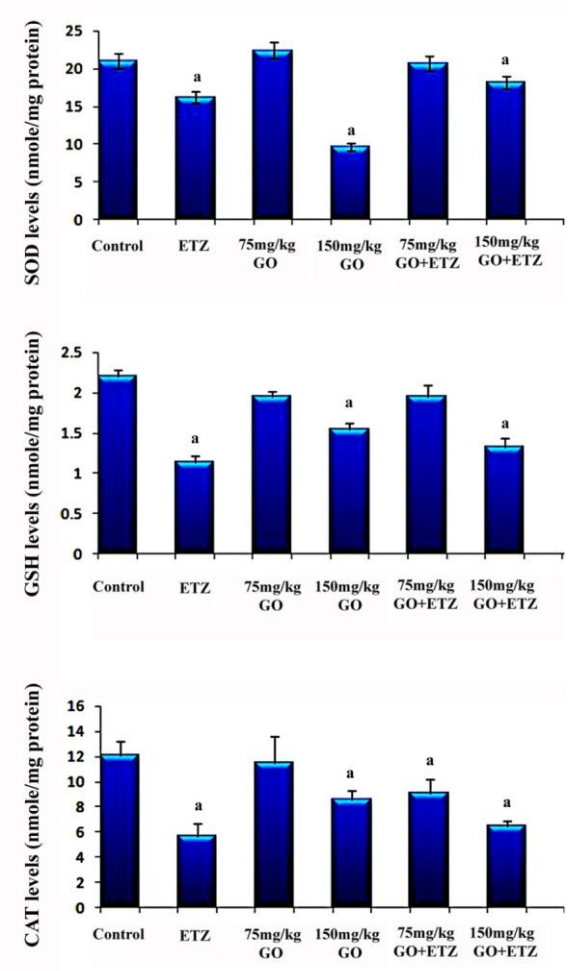

Fig-5: The protective effect of ginger oil (GO) treatments (75 &150mg/kg b.wt.) on antioxidants as measured as superoxide dismutase (SOD), catalase (CAT) activities and glutathione levels in spleen tissues of ETZ-treated rats. Data were represented as means of three independent experiments (n=5) and bars for standard deviation. ETZ, etoposide (1mg/kg b.wt.); GO, Ginger oil; a, statistically significant ($P \leq 0.05$) with respect to control.

DISCUSSION

Etoposide induced cellular DNA strand breaks by inhibiting topoisomerase II**.** This causes the DNA damage in both normal and cancer cells because it cannot diagnose the cancerous cell rather than the normal one [32].

The present study indicated that treatment of ETZ caused severe damage of DNA in spleen tissues. Combination with GO showed an improvement in DNA fragmentations after 21 days of treatment when compared with ETZ group. Similarly, ETZ caused apoptotic double strand DNA damage [33]. This may be due to reactive oxygen species production, lipid peroxidation, DNA intercalation, cross-linking and cell membrane damage in various tissues [34]. In this study, the higher doses of ginger oil caused damage in spleen tissues [35].

However, the results of leukocytes single cell gel electrophoresis (comet assay) revealed an increase in DNA damage of ETZ-treated group and the combination of GO higher dose. This may be due to the formation of reactive oxygen species (ROS), as superoxide anion and hydrogen peroxide that induced apoptosis through DNA cleavage. Oxidative stress by chemicals can serve as mediators of apoptosis [36]. The low dose of GO reduced the ETZ-induced DNA damage. Previous studies supported these findings at the levels of DNA damage and chromosomal aberrations [37]. Furthermore, ginger exerted anti-genotoxic effect against some anti-cancer drugs such as taxol by reducing the total chromosomal aberrations and high number of micronuclei [38].

The better ameliorated effect of DNA damage among the lower doses of GO in the current study can be attributed to the high amounts of antioxidants [39]. These antioxidant properties are due to its contents of gingerol, genistein, thymol and carvacrol [40].

This study revealed that serum MDA and NO levels were increased significantly in rats treated with ETZ in comparison with the control group. MDA and NO levels were significantly decreased in animals receiving GO in combination with ETZ.The results come in agreement with the results of Arun *et al.* [41] who indicated that ginger protects the spleen tissue

against lipid peroxidation at different doses.This may be due to its components of gingerol, zingerone, phenolic, 3-diketones and shogaol. They act as scavengers of oxygen radicals leading to the improvement of anti-oxidants levels [39] and protect against DNA and cellular damage [42]. The significant decrease in GSH levels, SOD and CAT activities relative to the control was evidenced in the present study. GO-receiving groups showed significant ameliorated effect.

Oxidative damage of DNA and tissues is considered the early manifestation of etoposide toxicity, which generates free radicals that caused depletion of antioxidants such as GSH, CAT and SOD [43]. Ginger increased the catalytic activity of catalase and improved the oxidative status in ETZ-treated rats.This may be due to its ability to scavenge free radicals and toxic carcinogenic electrophiles, therefore sparing the concentration of the endogenous antioxidant enzymes [42].

In agreement with our results, ginger ameliorated the SOD activity and GSH levels in hepatic tissues after lead acetate intoxication. It also increased glutathione peroxidase (GPX), glutathione-stransferase (GST) and CAT activities in renal tissues [44] and normalized lipid peroxidation by increasing SOD and CAT activities [45].

Our study indicated the protective effect of GO on spleen at low doses which protected the spleen from damage. However, the high dose showed slight damage in the spleen. These results are in agreement with Udo-Affah *et al.,* [46] who reported that the treatment with low and medium doses (100, 250mg/kg) of ginger extract produced little damaging effects in the spleen.

CONCLUSION

The pretreatment with GO resulted in a significant amelioration of oxidative status and DNA damage in ETZ-intoxicated animals. This suggest that GO has the potential to be used as a herbal remedy to reduce ETZ-induced genotoxicity and oxidative stress in albino rats.

Conflict of Interest

The authors declare that no conflict of interest regarding this article.

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