Original Article

The Protective Effect of Melatonin on Diazepam-induced Genotoxicity in Peripheral Blood Lymphocytes Using Micronucleus Assay

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Abstract

Background: Diazepam belongs to the benzodiazepine family of drugs and is mainly used for anxiety, muscle spasms, seizures, and insomnia. Long-term diazepam administration can cause genotoxicity, and oxidative stress is a likely molecular mechanism involved. **Objectives:** In this study, the benefits of melatonin against diazepam-induced oxidative damage and genotoxicity were investigated. **Materials and Methods:** Cultured peripheral lymphocytes were allocated to five groups: control, diazepam (100 µg/mL), melatonin (50 and 100 µM) with diazepam and cisplatin (0.05 µg/mL). After harvesting and preparing slides, the incidence of micronuclei (MN) was observed as a marker of genotoxicity. Then, in order to measure oxidative stress parameters, contents of glutathione (GSH) and lipid peroxidation (LPO) were determined. **Results:** Results documented increased MN and LPO and decrease in GSH levels in diazepam-administered lymphocytes versus those of the control group. When melatonin was given to diazepam-administered lymphocytes, they almost attenuated the increase of MN and LPO and restored the levels of GSH. **Conclusion:** Results showed that diazepam seems to induce genotoxicity in cultured human lymphocytes and oxidative stress plays an important role in it. Furthermore, it is concluded that melatonin efficiently protects against genotoxicity through its anti-oxidative effects.

Keywords: *Diazepam, genotoxicity, lymphocyte, melatonin, micronucleus, oxidative stress*

Introduction

Genotoxicity or genetic toxicity is the damaging effect of chemical and physical factors on DNA and genetic information of a living cell which results in mutations.[1] Many drugs and toxins can cause genotoxicity with different mechanisms, and one of the most important mechanisms involved is oxidative stress.[2] Oxidative stress is a state in which the balance between oxidant and antioxidant agents is disturbed in the cell, resulting from the release of free radicals and reactive oxygen species (ROS).[3] Antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione (GSH) S-transferase help the living cell fight ROS. ROS eventually attacks the cell's macromolecules, including proteins, lipid membranes, and DNA. In oxidative stress, the increase in ROS outweighs the cell's antioxidant defense mechanisms, which results in lipid peroxidation (LPO),[4] protein,[5] and DNA damage.[6]

Benzodiazepines are a group of drugs that affect the central nervous system (CNS) and have anxiolytic, sedative, hypnotic, anticonvulsant, and muscle relaxant properties.[7] Benzodiazepines bind to a specific site in the $GABA_A$ receptor and increase receptor affinity to gamma-amino butyric acid (GABA) neurotransmitter and thereby suppress the CNS. Diazepam belongs to the benzodiazepine family and has been used in the treatment of anxiety, symptoms of alcohol deprivation syndrome, muscle spasm, pre-operative anxiety, sedation in ICU patients, and seizures.[8]

There have been many studies on the genotoxicity of benzodiazepines such as bromazepam, chlordiazepoxide, diazepam, medazepam, midazolam, nitrazepam, oxazepam, alprazolam, and lorazepam over the years, and at least one positive result has been observed for each of these drugs.[1,9,10] Diazepam is widespread benzodiazepine, and recently its genotoxicity is considered as a major concern.[11-13] In addition, there have been studies that result in diazepam causing oxidative damage. For example, Azab *et al.*[14] found that diazepam induces oxidative genetic damage in cultured human lymphocytes. Another study showed that diazepam increases

How to cite this article: Soufi BR, Evazalipour M, Motavallian A, Chakosari MH, Zamani E. The protective effect of melatonin on diazepam-induced genotoxicity in peripheral blood lymphocytes using micronucleus assay. J Rep Pharma Sci 2022;11:92-7.

Received: 16 Sep 2020
Accepted: 22 Jan 2022
Published: 29 Jun 2022

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Access this article online

Website: www.jrpsjournal.com

DOI:10.4103/jrptps.JRPTPS_111_20

Quick Response Code:

LPO in the cerebral and cerebellar cortex and carbonyl protein formation in the rat brain striatum.[15] However, further studies are needed to confirm these results.

Antioxidants are substances that significantly inhibit or delay oxidizable substrates from oxidation. There have been several studies showing that antioxidants can inhibit DNA oxidative damage and genotoxicity by improving the protective effect of superoxidase, catalase, and GSH peroxidase enzymes against oxidative stress[16] and scavenging ROS from the cells.[17]

Melatonin is the main product of the pineal gland that adjusts the 24-h cycles and circannual rhythm regulation. Additionally, it modulates sleep disorders, immune function, and tumor growth inhibition and influences on retinal physiology.[18] It is known to be a potent cellular antioxidant. It can effectively scavenge free radicals and ROS[18,19] and increase activities of antioxidant enzymes including SOD, GSH peroxidase, and GSH reductase.[20] As a result, melatonin's antioxidant activity includes the protection of cell membrane lipids, cytosolic proteins, and nuclear DNA. The antioxidant activities of most molecules are limited due to their specific intercellular distribution. But melatonin, due to its small molecular size, can easily penetrate any subcellular part and cross all morphophysiological barriers and enter all cells of an organism.[21] Furthermore, melatonin has been shown to protect DNA from oxidative agents.[22,23] For example, Melchiorri *et al.*[23] showed that melatonin protected paraquat-induced genotoxicity in mice by its free radical scavenging activity.

Given the role of oxidative stress in the development of genotoxicity and as diazepam can cause oxidative stress, the use of antioxidants such as melatonin seems to be effective in preventing this toxicity. In contrast, results of studies on genotoxicity of diazepam are not consensual. Therefore, this study investigates the protective effect of melatonin in preventing genetic damage caused by diazepam in human lymphocytes using the micronucleus (MN) assay. The possibility of oxidative stress as a mechanism involved in the development of this toxicity is examined by measuring the contents of GSH and LPO.

Materials and Methods

Chemicals

RPMI-1640 medium, fetal bovine serum (FBS), antibiotic/antimycotic solution, phytohemagglutinin (PHA), diazepam, melatonin, cisplatin, cytochalasin B (Cyt-B), potassium chloride (KCl), methanol, glacial acetic acid, sucrose, magnesium chloride ($MgCl_2$), disodium phosphate (Na_2HPO_4), tris-hydrochloride (Tris-HCl), phosphoric acid, thiobarbituric acid (TBA), n-butanol, tetramethoxypropane, ethylenediaminetetraacetic acid, trichloroacetic acid (TCA), tris-ammonium, sodium acetate, dithionitro benzoic acid (DTNB), and GSH were used in this study. These chemicals were mainly purchased from Sigma–Aldrich (St Louis, MI, USA) and Merck (Darmstadt, Germany).

Cell preparation

Blood samples were taken from volunteers using syringes containing heparin under sterile conditions. The donors were healthy adult males who were non-smokers and non-drinkers and had not taken any particular medicine for a week before the samples were taken. All of the procedures of this study were approved by the Ethics Committee of Guilan University of Medical Sciences (IR.GUMS.REC.1398.050).

MN assay

MN assay is a comprehensive approach to assess genomic damage in cell cultures.[24] The assay was performed according to the method of Bolognesi and Fenech.[25]

Blood samples were seeded in the six-well plate with culture medium (RPMI-1640 medium, FBS 10%, and antibiotic/antimycotic solution). An aliquot of 100 µL PHA was added to each well in order to stimulate cell division. The plate was then transferred to the incubator at 37°C and 5% CO_2. Twenty-four hours later, the cultured peripheral blood lymphocytes were allocated to five groups: negative control (containing normal saline), diazepam (100 µg/mL),[14] melatonin (50 µM)[26] with diazepam, melatonin (100 µM)[27] with diazepam, and positive control (cisplatin 0.05 µg/mL).[28] Twenty hours after the exposure, cytochalasin-B was added to each well. Twenty-eight hours after adding cytochalasin-B, the cell culture media were harvested and centrifuged. Next, the supernatant was gently removed and the cells were treated with a mild hypotonic solution (0.075 M KCl for 5 min). After fixing the samples with glacial acid acetic solution, the cell suspensions staining was performed using 2% Giemsa. The frequency of MN was calculated per 1000 binucleated cells and reported as percentage.

Evaluation of oxidative stress parameters

LPO measurement

The content of malondialdehyde (MDA), a product formed as a result of LPO, was estimated using TBA as the indicator based on the reaction of TBA with MDA.[1] In brief, 0.25 mL sulfuric acid (0.05 M) was added to cell suspension from each experimental group. Next, all the microtubes were placed in boiling water bath for 30 min; afterwards, they were removed and left in an ice bath. Then, 4 mL of n-butanol was added to each microtube and mixed well. The microtubes were centrifuged at 3500 rpm for 10 min. The MDA content was assessed by measuring the absorbance in the n-butanol layer at 535 nm (Epoch™ Microplate Spectrophotometer, BioTek, USA). In this method, standard curve was calculated by tetramethoxypropane and MDA concentrations were expressed as µM.[29]

GSH content measurement

GSH content was evaluated using DTNB as the indicator, based on the formation of yellow color when DTNB reacts with compounds containing sulfhydryl groups. Briefly, 1 mL of each

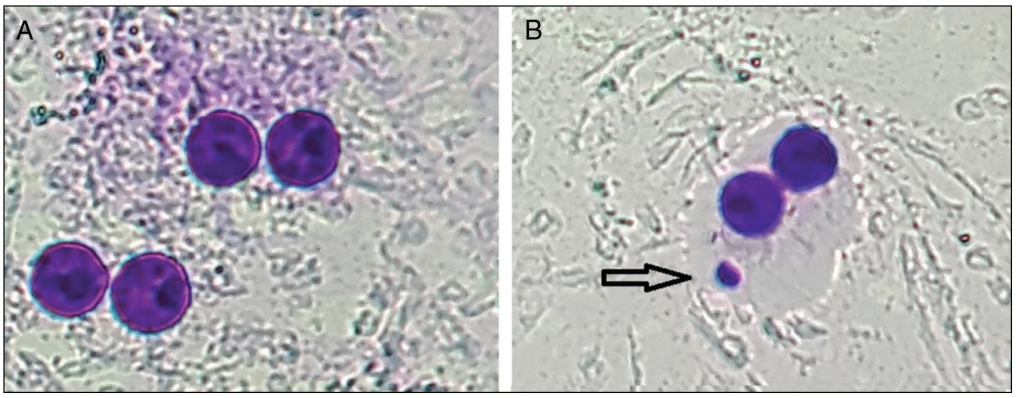

Figure 1: Photographs of binucleated human lymphocytes stained with Giemsa observed with an optical microscope (×1000): (a) without MN and (b) with a MN

sample was added to 1 mL TCA 10% and was mixed using a shaker for 2 min. Next, they were centrifuged for 15 min. An aliquot of 2.5 mL Tris buffer with pH 8.9 and 0.5 mL DTNB reagent were added to 1 mL of the supernatant. The resulting yellow color was read at 412 nm using a spectrophotometer. In this method, GSH was also used as a standard.[30]

Statistical analysis

Results are presented as mean ± standard deviation (SD). All statistical analyses were performed using GraphPad Prism (version 6). Statistical significance was determined using the one-way analysis of variance (ANOVA) test, followed by the *post hoc* Tukey test. Statistical significance was set at $P < 0.05$.

Results

Results of MN assay

The frequency of MN in binucleated human lymphocytes after treatment with diazepam, melatonin, and cisplatin was measured [Figure 1]. As shown in Figure 2, the frequency of MN in lymphocytes treated with diazepam was 9.25 ± 1.71, which is a significant increase compared with the control group ($P < 0.001$). This can be considered a sign of the genotoxicity of diazepam. Furthermore, frequency of MN in lymphocytes treated with diazepam and melatonin 50 and 100 µM was 4.5 ± 1.3 and 3.25 ± 0.5, respectively, which was significantly decreased when compared with the diazepam group ($P < 0.001$). This is indicative of the protective effect of melatonin on the genotoxicity of diazepam. MN frequency in the diazepam and melatonin 50 µM group showed a significant difference when compared with the control group ($P < 0.05$), but this difference was not significant in the diazepam and melatonin 100 µM group when compared with the control group. This may indicate that an increase in melatonin concentration is directly linked to an increase in its protective effect on diazepam genotoxicity.

Results of LPO measurement

As shown in Figure 3, the concentration of MDA in blood samples treated with diazepam was 7.54 ± 2.2, which has increased significantly when compared with the control group

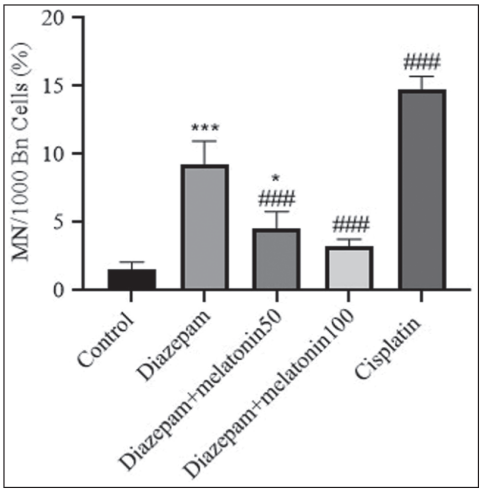

Figure 2: Frequency of MN in binucleated human lymphocytes treated with diazepam (100 µg/mL), melatonin (50 and 100 µM), and cisplatin (0.05 µg/mL). Values represent mean ± SD, ***$P < 0.001$ compared with the control group, *$P < 0.05$ compared with the control group, ###$P < 0.001$ compared with the diazepam group

($P < 0.001$). This can be considered a sign of oxidative stress caused by diazepam. Furthermore, MDA concentration in blood samples treated with diazepam and melatonin 100 µM was 3.44 ± 0.77, which has decreased significantly when compared with the diazepam group ($p < 0.01$). This could indicate that melatonin can protect against the oxidative stress caused by diazepam. MDA concentration in samples treated with diazepam and melatonin 50 µM was 7.077 ± 0.62, which has increased significantly when compared with the control group ($P < 0.001$), whereas only negligible reduction has been observed when compared with the diazepam group. Therefore, it seems that melatonin has a significant protective effect on diazepam oxidative stress by reducing LPO at concentrations above 50 µM.

Results of GSH content measurement

As shown in Figure 4, the concentration of GSH in diazepam-treated blood samples was significantly lower than that of the control group ($P < 0.001$). This can be considered a sign of the oxidative stress caused by diazepam. Furthermore, GSH concentration in samples treated with diazepam and melatonin

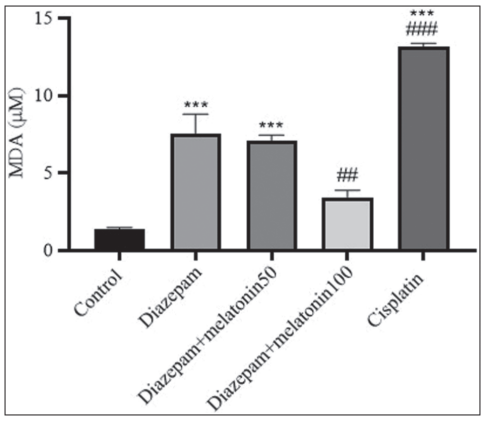

Figure 3: A comparison of MDA concentrations in the blood samples treated with diazepam (100 μg/mL), melatonin (50 and 100 μM), and cisplatin (0.05 μg/mL). Values represent mean ± SD, ***$P < 0.001$ compared with the control group, ##$P < 0.01$ compared with the diazepam group, ###$P < 0.001$ compared with the diazepam group

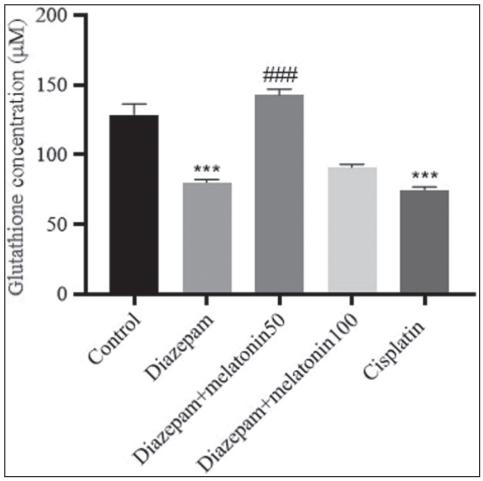

Figure 4: A comparison of GSH concentrations in the blood samples treated with diazepam (100 μg/mL), melatonin (50 and 100 μM), and cisplatin (0.05 μg/mL). Values represent mean ± SD, ***$P < 0.001$ compared with the control group, ###$P < 0.001$ compared with the diazepam group

50 μM was significantly higher than that of the diazepam group ($P < 0.001$), and even the highest concentration of GSH belonged to this group. This may be an indication of the protective effect of melatonin on oxidative stress induced by diazepam. GSH concentration in samples treated with diazepam and melatonin 100 μM was not significantly higher when compared with that of the diazepam group and was even less than that of the melatonin 50 μM group.

Discussion

Different compounds can cause genotoxicity, or in other words damage to the genetic material, with different mechanisms. The induction of genotoxicity through oxidative damage to DNA by oxidant compounds is well documented.[2,31,32] In this study, genotoxicity of diazepam was investigated in human blood lymphocytes using the MN test. The results of this test showed that treating lymphocytes with 100 μg/mL diazepam resulted in a significant increase in the frequency of MN.

A number of studies have investigated the genotoxicity of diazepam. As an example, a dose-dependent increase in sister chromatid exchanges (SCE) and a significant decrease in proliferation rate index were observed in the cultures of human peripheral blood lymphocytes receiving diazepam (2.6–211.17 μM).[33] These results confirm the results of the current study regarding the genotoxic effect of diazepam in the culture of human lymphocytes, with the difference that a higher concentration (351 μM) has been implemented in this study. In another study, in SCE and chromosomal aberration tests, exposing cultured lymphocytes to diazepam (1, 10, and 100 μg/mL) could cause dose-dependent oxidative DNA damage in the 8-hydroxy-deoxyguanosine test.[14] Despite these studies, the authors called for further studies to confirm the genotoxicity of diazepam. Accordingly, the current study can validate this conclusion.

Much researches have been done to determine the role or involvement of oxidative processes in the brain through free radicals after diazepam administration.[34,35] Studies have shown that diazepam causes tissue oxidative damage, especially after long periods of treatment. As an example, Castro *et al.*[15] showed that diazepam (1 mg/kg) increases LPO in the cortex and cerebellum and induces the formation of carbonyl protein in the striatum of mouse brain. In another study, a significant decrease in GSH levels and SOD activity and a significant increase in LPO and DNA synthesis in the liver of rats treated with diazepam (3 mg/kg) were observed.[36]

In this study, in order to investigate oxidative stress as one of the possible mechanisms involved in the genotoxicity of diazepam, MDA and GSH concentrations in human blood lymphocyte cultures were measured. The results showed that lymphocytes treated with 100 μg/mL diazepam had a significant increase in MDA concentration and a significant decrease in GSH concentration. It can be concluded that diazepam with a final concentration of 100 μg/mL induces oxidative stress through LPO and through decreasing GSH concentration in human blood lymphocytes.

There are several studies that show that antioxidants can inhibit oxidative stress and genotoxicity.[37-39] Melatonin is known to be a potent cellular antioxidant. Numerous studies have examined protection of melatonin against the genotoxicity of various drugs and substances. For example, one study that evaluated the protection of melatonin (100, 200, 300, and 400 μM) against genotoxicity of diazinon (an organophosphorus insecticide) using the MN test in human blood lymphocytes showed that melatonin significantly decreases MN frequency in diazinon-receiving lymphocytes depending on dose. This suggests that melatonin has a potent and dose-dependent effect against DNA damage caused by diazinon.[27]

In this study, the protective effect of melatonin on the genotoxicity of diazepam using the MN test *in vitro* was examined. The results of this test showed that adding 50 μM melatonin to diazepam-treated lymphocytes significantly reduces the frequency of MN. Moreover, by increasing the

dose of melatonin to 100 μM, the positive effect of increasing melatonin concentration on protection against diazepam genotoxicity was observed. Finally, it can be concluded that melatonin has a significant and dose-dependent protective effect on the genotoxicity of diazepam in human blood lymphocytes.

MDA and GSH concentrations were also measured to assess the protective effect of melatonin on oxidative stress caused by diazepam. Summarizing the results of these two tests, it can be said that melatonin has a protective effect on oxidative stress caused by diazepam by reducing MDA concentration and increasing GSH concentration. The difference is that this decrease in MDA concentration due to melatonin is significant at its higher concentration (100 μM), but this increase in GSH concentration is significant at its lower concentration (50 μM).

A number of studies have been performed *in vivo* to investigate the effect of melatonin on oxidative stress, and these studies demonstrate the antioxidant properties of melatonin against oxidative stress.[40-42] For example, the results of one study showed an increase in DNA synthesis and LPO and a decrease in GSH levels and SOD activity in the liver of rats treated with diazepam (3 mg/kg). Next, when melatonin (5 mg/kg) was added, it attenuated this increase in DNA synthesis and LPO and restored GSH levels and SOD activity.[36] In another study, melatonin (0.1 mg/kg/day) decreased the elevated LPO, GSSG, and phosphatase acid caused by an acute treatment with cyclophosphamide in mice. Reduction in GSH, GSH peroxidase, and alkaline phosphatase due to cyclophosphamide was also amended by chronic oral administration of melatonin.[43]

Based on the results, it seems that the protective effect of melatonin on oxidative stress by reducing LPO is observed more in high concentrations of melatonin, and increasing the concentration of melatonin has a positive effect on its protective effect (whereas in the GSH concentration assay, the lower concentration of melatonin was more effective). However, it can be said that melatonin has a protective effect on oxidative stress caused by diazepam via different mechanisms.

Conclusions

This study showed that diazepam is able to induce genotoxicity in healthy blood lymphocytes, and oxidative stress is an important mechanism involved. It was also concluded that melatonin ameliorates diazepam-induced oxidative stress by reducing LPO and increasing GSH concentration and has a protective effect on the genotoxicity of this drug in human blood lymphocytes.

Acknowledgments

The data provided in this study were extracted from a part of Pharm D. Thesis of Bahar Rezaei Soufi and supported by the Research Council of Guilan University of Medical Sciences, Rasht, Iran (IR.GUMS.REC.1398.050).

Financial support and sponsorship

This research was funded by the Research Council of Guilan University of Medical Sciences, Rasht, Iran.

Conflicts of interests

The authors have no conflict of interests to declare.

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