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Diazepam induced oxidative DNA damage in cultured human lymphocytes

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ABSTRACT

Diazepam is a benzodiazepine compound that is mainly used for anxiety, muscle spasms, seizures and insomnia. Several studies have shown that long-term Diazepam treatment is associated with oxidative stress. In this study, the possible genotoxic effect of Diazepam was examined in cultured human white blood cells using the sister chromatid exchanges (SCEs), chromosomal aberrations (CAs) and 8-hydroxy-deoxyguanosine (8-OHdG) assays. Treatment of cultured lymphocytes with different concentrations of Diazepam (1, 10 and 100 μg/mL) did not induce chromosomal DNA damage as measured using SCEs and CAs assays ($P > 0.05$). In addition, no effect was observed on mitotic and proliferative indices ($P > 0.05$). However, Diazepam induced oxidative DNA damage as measured by the 8-OHdG assay in a dose dependent manner ($P < 0.001$). In conclusion, Diazepam seems to induce oxidative DNA damage in cultured human lymphocytes. More *in vivo* studies are required to confirm current finding.

1. Introduction

Diazepam belongs to benzodiazepine family medications with anticonvulsant, anti-anxiety, and skeletal muscle relaxing properties. Diazepam is considered as one of the most prescribed medication worldwide and is recommended by World Health Organization as a core medicine for treatment of several diseases such as anxiety, seizures, alcohol withdrawal and insomnia (Calcaterra and Barrow, 2014). The mechanism of action of Diazepam involves enhancement of the action of the neurotransmitter GABA via interacting with the benzodiazepine site on the GABA receptor, and the subsequent depression of the central nervous system (Calcaterra and Barrow, 2014; Sakai and Ishizuka, 2009). The advantages of diazepam include its rapid and potent effects compared to other similar medications. However, higher doses of Diazepam have been shown to be associated with anterograde amnesia sedation, excitement, worsen depression, physical dependence, benzodiazepine withdrawal syndrome and cognitive impairments (Duley et al., 2010).

Recently, Diazepam has been shown to cause oxidative tissue damage, especially during long periods of treatments. For example, Castro et al. (2009) showed that treatment of Diazepam increased lipid peroxidation in the cortex and cerebellum, and increased protein carbonyl formation in the striatum of mouse brain. Rats administered Diazepam showed significant decreases in glutathione levels and superoxide dismutase activity in liver (El-Sokkary, 2008). In this study, we proposed to examine the effects of Diazepam on inducing DNA damage using 8-hydroxy-deoxyguanosine (8-OHdG) assay in cultured human white blood cells. 8-OHdG is an oxidative stress biomarker that has been shown to increase in conditions and diseases that marked by elevation in oxidative stress (Kroese and Scheffer, 2014). We also examined genotoxic effect of Diazepam using sister chromatid exchanges (SCEs) and chromosomal aberrations (CAs) assays as they also can be modulated by exposure to mutagenic agents including drugs that can generate free radicals inside cells (Norppa et al., 2006).

2. Materials and methods

2.1. Subjects

Five volunteers were recruited in the study to donate blood for lymphocyte cultures. The donors were healthy non-smokers and

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non-alcoholic adult males (20–26 year old). About 20 mL blood was collected from each subject in heparinized collection tubes. Prior to start of the study, written informed consents was obtained from all subjects according to Institutional Review Board.

2.2. Chemicals

Diazepam, Cisplatin, Bromodeoxyuridine, DMSO and Colchicine were obtained from Sigma–Aldrich (Saint Louis, MI, USA). The 8-OHdG assay kit was purchased from Cayman Chemical (Ann Arbor, Michigan, USA). Finally, Pb-Max Culture media was obtained from Gibco-Invitrogen (United Kingdom).

2.3. Cell cultures

Blood lymphocytes cultures were initiated by adding 1 mL of freshly withdrawn blood into tissue-culture flask containing 9 mL of complete lymphocyte Pb-Max media (RPMI 1640 medium supplemented with suitable amount of fetal bovine serum, glutamine, Penicillin-Streptomycin and Phytohaemagglutinin). Diazepam was dissolved in Dimethyl sulfoxide. Experimental cultures were treated with Diazepam at a final concentration of 1, 10 and 100 μg/mL (equivalent to 3.5, 35 and 351 μM respectively) whereas control cultures were treated with vehicle (Dimethyl sulfoxide). These concentrations were selected based on previous studies that showed positive results equivalent ones (Akritopoulou et al., 2009).

2.4. Sister-chromatid exchange assay

Details of the procedure used for the detection of SCEs in cultured lymphocytes were as previously described (Alsatari et al., 2012; Khabour et al., 2011). In brief, Bromodeoxyuridine solution was freshly prepared in distilled sterile water and was added to a final concentration of 20 μg/mL directly after culture initiation. Cultures were incubated at 37 °C in CO_2 incubator for 72 h. Diazepam was added to cultures in the last 24 h of incubation time. As a positive control, Cisplatin (1 μg/mL, final concentration) was used and was added in the last 24 h of incubation time. Before harvesting of cultured lymphocytes, Colchicine (finale concentration 10 μg/mL) was added to cultures for 2 h. Cultures were then centrifuged at 1000×*g* for 5 min, decanted and the cellular pellet was gently re-suspended in 10 mL hypotonic solution (0.075 M KCl) at 37 °C for 20 min. The cellular suspension was centrifuged at 1000×*g* for 5 min and the cellular pellet was fixed with three changes of ice-cold methanol: acetic acid (3:1). The cellular suspension was then dropped on pre-chilled microscope slides to obtain metaphase spreads. The slides were stained with the fluorescent-plus-Giemsa technique as described previously (Azab et al., 2009). The slides were analyzed blindly using medical microscope at 1000 × magnification. About 250 M2 metaphase spreads (50 from each donor) were analyzed per each drug concentration for presence of SCEs (Alzoubi et al., 2014a; Khabour et al., 2016).

2.5. Chromosomal aberrations (CAs) assay

Blood lymphocytes cultures were initiated by adding1 mL of freshly withdrawn blood into tissue-culture flask containing 9 mL of complete lymphocyte Pb-Max media. Cultures were incubated at 37 °C in CO_2 incubator for 72 h. Drugs were added to cultures in the last 24 h of incubation time. Colchicine (finale concentration 10 μg/mL) was added to cultures for 2 h prior to harvesting period. Cultures were then centrifuged at 1000×*g* for 5 min, decanted and the cellular pellet was gently re-suspended in 10 mL hypotonic solution (0.075 M KCl) at 37 °C for 20 min. The cellular suspension was centrifuged at 1000×*g* for 5 min and the cellular pellet was fixed with three changes of ice-cold methanol: acetic acid (3:1). The cellular suspension was then dropped on pre-chilled microscope slides to obtain metaphase spreads. The slides were stained with 2% giemsa solution (pH 6.8) for 15 min and subsequently analyzed for presence of CAs analysis (Khabour et al., 2015). The slides were analyzed blindly using medical microscope at 1000× magnification. About 500 well-spread metaphases (100 from each donor) were scored for the presence of CAs. Only breaks and exchanges were included in the analysis (Alzoubi et al., 2012).

2.6. 8-OHdG assay

The 8-OHdG assay was performed as previously described (Khabour et al., 2014). In brief, blood cultures were incubated for 72 h at 37 °C. Cultures were then washed 5 times using RPMI medium. Since fetal bovine serum is very rich in 8-OHdG, Cells were then re-suspended in RPMI medium supplemented with only glutamine, Penicillin-Streptomycin and Phytohaemagglutinin. Cultures were then treated with drugs (Diazepam or positive control) and incubated at 37 °C for 6 h. Cultures were then centrifuged at 1000×*g* and 200 μL from the supernatant were used for 8-OHdG assay. Competitive ELISA assays for 8-OH-dG was performed according to the manufactures protocol. ELISA plate were read at 405 nm using an automated reader (ELx800 Universal microplate reader, BIO-TEK, USA). The period of treatment with Diazepam was reduced to 6 h in this assay compared with 24 h in the case of SCEs and CAs to avoid deteriorations of cultures due to the absence of fetal bovine serum. This assay has been to be potent to examine the effects of oxidative DNA damaging agent on cultured human lymphocytes (Alzoubi et al., 2014b; Azab et al., 2016; Esmadi et al., 2016; Mhaidat et al., 2016).

2.6. Cell kinetics analysis

The cytotoxic effect of drugs was examined using both the mitotic index and proliferative index. The mitotic index was determined by scoring at least 5000 cells (1000 cells from each donor) and counting the cells that were in metaphases. For the cell proliferation index, 500 metaphase cells were used. The proliferation index was calculated using the following formula = (1 × [M1] + 2 × [M2] + 3 × [≥M3])/100, where M1, M2 and M3 are the number of cells at the first, second and third metaphase, respectively (Alzoubi et al., 2014b).

2.7. Statistical analysis

All data were expressed as mean ± SEM. Graph Pad Prism software (version 5) was used for statistical analysis that includes ANOVA followed by Tukey posthoc test. A $P < 0.05$ was considered significant.

3. Results

The genotoxic effects of Diazepam on blood lymphocytes were evaluated using three assays. In the CAs and SCEs assays, cultures were treated with the different concentrations of diazepam in the last 24 h of incubation period. Fig. 1A shows changes in the levels of CAs induced by the treatment. None of the examined Diazepam concentrations (1, 10 and 100 μg/mL) induced elevation in the frequency of CAs ($P = 0.93$, ANOVA $F = 0.1401$). Similar results were obtained with the SCEs assay as shown in Fig. 1B. None of the examined Diazepam concentrations affected SCEs frequency observed in the control group ($P = 0.326$, ANOVA F value = 1.156). The positive control, Cisplatin, significantly increased the frequency of SCEs (10.56 ± 0.66 in Cisplatin group versus 4.87 ± 0.21 in the control group, $P < 0.001$) and CAs (0.092 ± 0.013 in cisplatin