DOI: 10.4149/BLL_2012_079

Bratisl Lek Listy 2012; 113 (6)

350 – 353

EXPERIMENTAL STUDY

Protective role of vitamin C in diazepam-induced apoptosis in rat thymocytes

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Abstract: Diazepam, a peripheral-type benzodiazepine receptor ligand, is widely used as a therapeutic agent. On the other hand, peripheral-type benzodiazepines have been shown to induce apoptosis in different immune cell types. In this study, we examined the possible protective role of vitamin C in diazepam-induced apoptosis and evaluated the cellular content of glutathione during this process. Rat thymocytes were incubated for 24 hours with diazepam and increasing concentrations of vitamin C or with diazepam alone. The exposure to diazepam resulted in an increase in apoptotic cell death and decrease in glutathione content in rat thymocytes. Vitamin C was effective in ameliorating the effect of diazepam in rat thymocytes by decreasing the proportion of apoptotic cells and increasing the cellular content of glutathione. These results suggest that vitamin C reduced the diazepam-induced apoptosis in rat thymocytes by restoring the cellular content of glutathione, which may be useful in preventing the diazepam-induced immunosupression *(Tab. 1, Fig. 1, Ref. 31)*. Full Text in PDF *www.elis.sk.*
Key words: vitamin C, diazepam, thymocytes, apoptosis, glutathione.

The benzodiazepines are a group of psychoactive drugs that exert a number of pharmacological effects such as sedation, anxiolysis, hypnosis, anticonvulsant activity and muscle relaxation. Their physiological effects are mediated by binding to the central benzodiazepine receptor, which is associated with γ-aminobutyric acid $(GABA)_A$ receptor in the central nervous system. Benzodiazepines also bind to other receptors located mainly in peripheral tissues. The latter receptors are called peripheral benzodiazepine receptors (PBRs) (8). PBR is an 18 kD protein localized on the outer mitochondrial membrane in a wide variety of cell types, including the cells of the immune system (29). Although its precise function still remains unknown, PBRs have been associated with several mitochondrial functions such as calcium channel activity, anion transport, and modulation of mitochondrial membrane potential (3). A decrease in mitochondrial transmembrane potential (MTP) has been implicated as a critical effector of apoptosis in different cell types (25) and thymocytes (11). MTP is controlled by mitochondrial permeability transition pore (MPT) which contains PBR as its component (10). Further, antiapoptotic protein, Bcl-2, maintains MPT closure by binding to mitochondrial PBR (25), indicating a significant role of PBR in apoptosis induction. These observations were confirmed in a previous study showing that rat thymocytes treated with diazepam resulted in increased apoptosis rate and decreased MTP (24). This may partially explain the earlier described immunosuppressive effects of benzodiazepine (21).

Vitamin C (ascorbic acid) is a water-soluble and versatile physiological antioxidant that quenches reactive oxygen species (ROS) in both extracellular and intracellular compartments (12). As an effective scavenger, , it can react *in vivo* with superoxide ions, hydroxyl ions, hydrogen peroxide and water-soluble peroxyl radicals thus protecting the DNA, proteins and lipids from oxidation by ROS (6). Various studies have demonstrated that the treatment with diazepam (a peripheral-type benzodiazepine receptor ligand) induces ROS-mediated changes in different cell types with decreased cellular content of glutathione (15, 7). The protective effect of vitamin C on diazepam-induced oxidative stress has been documented in liver (7) but has not been studied in thymus. Therefore, the current study was design to evaluate the possible protective role of vitamin C in diazepam-induced cytotoxicity in rat thymus and to answer the question whether this process involves the changes in cellular content of glutathione. nnnn

Material and methods

Culture medium (CM) was prepared using RPMI 1640 (Sigma, St Louis, Mo., USA), according to the manufacturer's instructions. CM containing 25 mM HEPES, 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) and 10 % fetal calf serum (FCS).

Propidium iodide (PI) was purchased from Santa Cruz Biotechnology, Santa Cruz, USA.

Vitamin C (L-ascorbic acid) and diazepam were obtained from Galenika a.d., Belgrade, Serbia.

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Acknowledgements: This work was supported by the Ministry of Science and Environmental Protection of Serbia (Project 145081).

The 5-chloromethylfluorescein diacetate (5CMF-DA) was purchased from Molecular Probes, Eugene, OR, USA.

Animals

Experiments were performed on 8-10-week old adult male Wistar rats (140–160 g) bred at the Vivarium of Institute of Biomedical Research, Medical Faculty, Nis, under conventional laboratory conditions. The experimental animals were treated in accordance with national animal protection guidelines.

Preparation of thymocytes

Thymocytes were isolated in a manner described above (18). In brief, the thymus was dissected under aseptic conditions and placed in cold CM containing 10 % FCS. The thymocytes were released by sliding the thymus along the steel-mesh. Cell suspensions were filtered through a sterile nylon filter to remove the stroma and then the cells were washed twice with cold CM containing 10 % FCS. The viability of freshly isolated thymocytes determined by trypan blue dye exclusion method was always over 95 %.

Cell culture

Isolated thymocytes ($1x10^6$ cells/well; 200 µl) were cultivated in round-bottom 96-well plates (NUNC, Aarhus, Denmark). Cells were treated with increasing concentrations of vitamin C (ranging from 10 to 1000 µg/ml) and diazepam (25 µM), diazepam alone (25 µM), vitamin C alone (10–1000 µg/ml) or in CM alone (controls). All cell cultures were done in triplicates and incubated in an incubator (Galaxy, Wolf laboratories, USA) at 37 °C for 24 hours in atmosphere of 95 % air and 5 % carbon dioxide. The concentrations of diazepam, vitamin C, as well as the incubation time used in this study, were adapted from previous reports (24, 2) on the effect of these compounds on thymocytes toxicity.

Apoptotic DNA analysis

Thymocytes undergoing apoptosis were identified by their reduced relative nuclear DNA content as previously described (16). Single apoptotic cells were detected using a flow cytometer (Coulter XL-MCL) as a reduction in fluorescence of the DNA-binding dye PI in apoptotic nuclei. The percentage of apoptotic cells (subdiploid DNA peak in DNA fluorescence histogram) was determined.

Measurement of cellular content of glutathione (GSH)

The cellular content of GSH in thymocytes was estimated by 5-chloromethylfluorescein diacetate (5CMF-DA) as previously described (4). It has been documented earlier that the relation between the intensity of 5CMF-DA fluorescence and cellular content of glutathione is direct with a correlation coefficient of 0.965 between them (4). GSH levels were detected with a Perkin-Elmer fluorimeter (Wallac Victor^{2}V, Turku, Finland) while using an excitation wavelength of 480 nm and fluorescence emission collected at 525 nm. For each sample, basal fluorescence intensity values were subtracted from those obtained after different treatments and the results were presented as the ratio of mean fluorescence intensity (MFI).

Tab. 1. *In vitro* effect of diazepam and vitamin C on rat thymocytes apoptosis.

Culture conditions	Apoptosis (%)
Medium	35.3±3.53
Diazepam	43.2±3.11#
Diazepam+VC1	21.5±4.66**
Diazepam+VC2	29.45±2.47*
Diazepam+VC3	34.8±4.52

Rat thymocytes ($1x10^6$ cells/well) were cultured for 24h with increased concentrations of vitamin C and diazepam or with diazepam alone. Apoptosis was measured by flow cytometry as described in section Material and methods. The results are presented as mean percentage ± SD. Abbreviations: Diazepam+VC1 – cells treated with diazepam and vitamin C (1000 µg/ml), diazepam+VC2 – cells treated with diazepam and vitamin C (100 µg/ml), Diazepam+VC3 – cells treated with diazepam and vitamin C (10 µg/ml). # p<o.o5 compared to medium control (non-treated) cells, * p<0.05, ** p<0.01 compared to diazepam-treated cells.

Statistical analysis

All values are expressed as mean ± SD. The comparisons among groups were carried out using the Student t test and analysis of variance (ANOVA) coupled to the Dunnett's post hoc test. A p value <0.05 was considered significant.

Results

To investigate the effect of diazepam on rat thymocytes apoptosis, the cells were cultured with diazepam for 24 hours and assayed for apoptosis. *In vitro* exposure to diazepam resulted in significantly increased rat thymocyte apoptosis (Tab. 1). The obtained results presented in Table 1 show that an increasing concentration of vitamin C (100, 1000 µg/ml) significantly decreased the diazepam-induced rat thymocytes apoptosis, while the lowest concentration of vitamin C (10 µg/ml) failed to induce a significant reduction in apoptosis rate.

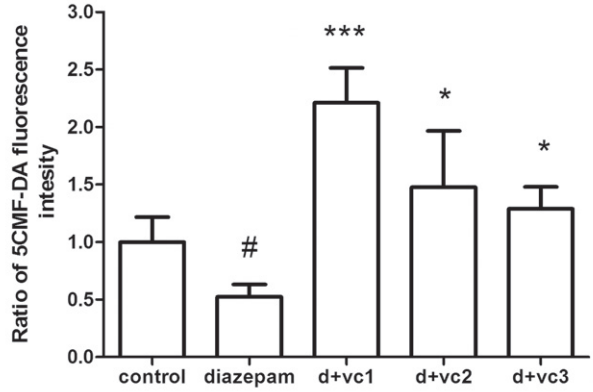

Fig. 1. Effects of diazepam and vitamin C on 5CMF-DA fluorescence intensity (cellular glutathione content) in rat thymocytes. Cells ($1x10^6$ cells/well) were treated with increasing concentrations of vitamin C and/or diazepam for 24 hours. The 5CMF-DA fluorescence intensity (cellular glutathione content) was measured using a fluorimeter as described in section Material and methods. Results are given as ratio of MFI ± SD of triplicate samples. Abbreviations: d+vc1 – cells treated with diazepam and vitamin C (1000 µg/ml), d+vc2 – cells treated with diazepam and vitamin C (100 µg/ml), d+vc3 – cells treated with diazepam and vitamin C (10 µg/ml). # p<0.05 compared to control (non-treated) cells, *p<0.05, ***p<0.001 compared to diazepam-treated cells.

In an attempt to gain an insight into the mechanisms by which diazepam exerts its apoptotic effect, the cellular content of glutathione was measured. The incubation of rat thymocytes with diazepam resulted in a significantly lower 5CMF-DA fluorescence intensity, thus ndicating a decrease in cellular content of glutathione by 25 μM diazepam (Fig. 1). The concentration-dependent effect of vitamin C on mean intensity of 5 CMF-DA fluorescence of rat thymocytes was examined when the cells were treated simultaneously with diazepam and vitamin C. Figure 1 shows that increasing concentrations of vitamin C (10–1000 μg/ml) produced a significant concentration-dependent increase in the intensity of 5CMF-DA fluorescence, thus indicating that vitamin C increased the cellular content of glutathione in rat thymocytes. Similar results were obtained in cell cultures treated only with vitamin C (data not shown).

Discussion

In the immune and hematopoietic systems, cell death is regulated by various apoptosis-stimulating pathways. Some pathways involve an early disorganization of mitochondria associated with a production of ROS and resultant oxidative stress (9). The induced oxidative stress can increase the cytosolic calcium concentration which further irreversibly opens MPT and leads to MTP loss, release of cytochrome c, and inhibition of mitochondrial respiration (17).

The current study results showed that diazepam, a peripheral-type benzodiazepine receptor ligand, was able to induce apoptosis in rat thymocyte cultures. Furthermore, micromolar diazepam concentration decreased the intensity of 5CMF-DA fluorescence, thus indicating that diazepam induced a decrease in cellular content of glutathione. The diazepam-induced rat thymocytes apoptosis associated with the collapse of MTP has been confirmed earlier (24), as well as the genotoxic effects of diazepam in human lymphocytes (1). On the other hand, there is increasing evidence suggesting that the toxicity of diazepam is mediated by oxidative stress. Diazepam administration increased the levels of lipid peroxidation and decreased glutathione and superoxide dismutase levels in brain (15) and liver tissues (7). Taken together with our results, it appears that the activation of PBR leads to disrupting the MTP and reducing the intracellular glutathione content in rat thymocytes, which may have an important role in the development of oxidative stress and resulting cytotoxicity.

Based on previous findings, we hypothesized that ascorbic acid could modulate diazepam-induced toxicity in rat thymocytes. Increasing concentrations of ascorbic acid significantly decreased the apoptosis rate in rat thymocytes, thus indicating a protective role of ascorbic acid in diazepam-induced thymus cytotoxicity. In addition, the ascorbic acid concentration dependently attenuated the diazepam-induced decrease in cellular content of glutathione. These findings correlate with decreased thymocytes apoptosis, suggesting that ascorbic acid attenuates the oxidative stress in rat thymocytes. The preventive role of ascorbic acid in diazepam-induced oxidative stress has been confirmed recently in liver (7) while herein we demonstrated the protective role of ascorbic acid in thymocytes. Ascorbic acid as an antioxidant presents in extracellular fluid and cytosolic compartment of cells while exerting several diverse effects on the immune system cells including T lymphocytes (14), polymorphonuclear leukocytes and macrophages (5). It has been shown that ascorbic acid enhances antioxidant defenses of T cells (27) and inhibits various forms of T-cell death (2). Glutathione and ascorbic acid show a strong *in vivo* functional interdependence (28). It is well documented that ascorbic acid supplementation ameliorated the dysfunction associated with glutathione deficiency (13, 7). By showing that ascorbic acid could elevate glutathione levels through the pentose phosphate pathway, it has been previously documented that the relationship between ascorbic acid and glutathione was inverse (22). On the other hand, several studies showed that ROS sensitize the T cells (9, 19) to apoptosis by decreasing the expression of Bcl-2 protein, which may result in mitochondrial dysfunction by unbinding the Bcl-2 protein from mitochondrial PBR (25). Such observations are in accordance with the findings that ascorbic acid in various cell types (23) and thymocytes (20) up-regulates the Bcl-2 protein expression. These findings may suggest that the imbalance between prooxidant and antioxidant systems in thymocytes after diazepam treatment can be restored by ascorbic acid. Immune cells are particularly sensitive to oxidative stress because of high content of polyunsaturated fatty acids in their plasma membranes and high ROS production, which is part of their normal function (26). By detoxifying ROS, antioxidants may therefore reverse the ROS-induced decline in Bcl-2 and prevent the cellular death (9, 30, 31). It should be noted that these results were obtained *in vitro* conditions, using cells from animals endogenously producing ascorbic acid. However, the evaluation of the *in vivo* protective effect of ascorbic acid, as well as the use of thymocytes from animals dependent on exogenous ascorbic acid could resolve and confirm the protective role of ascorbic acid. Nevertheless this hypothesis requires further studies.

In summary, we have shown that *in vitro* exposure to diazepam resulted in an increase in thymocytes apoptosis and reduction in intracellular glutathione content. Administration of antioxidants such as ascorbic acid which reduced the apoptosis rate of thymocytes and restored the cellular glutathione content may be useful in preventing the diazepam-induced immunosupression.

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Received February 2, 2010.
Accepted February 20, 2012.