Melatonin and vitamin C administration ameliorate diazepam-induced oxidative stress and cell proliferation in the liver of rats

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Abstract. *Objective:* Oxidative stress is a likely molecular mechanism in long-term diazepam administration. The benefits of antioxidants (melatonin and vitamin C) against diazepam-induced cell proliferation, DNA synthesis and oxidative damage were investigated in this study. *Materials & methods:* Four equal-sized groups of male rats [control, diazepam (3 mg/kg), diazepam plus melatonin (5 mg/kg) and diazepam plus vitamin C (50 mg/kg)] were used. Levels of lipid peroxides (LPO), superoxide dismutase (SOD) activity and glutathione (GSH) concentration were measured in tissue homogenates. Cell proliferation and rate of DNA synthesis were detected by autoradiography. *Results:* Results documented increased labelling index, ³H-thymidine incorporation (DNA synthesis), LPO plus decrease in GSH levels and SOD activity in livers of diazepam-administered rats versus those of controls. When melatonin and vitamin C were given to diazepam-administered rats, they almost attenuated the increase of labelling index, DNA synthesis and LPO, and restored the levels of GSH and SOD activity. *Conclusion:* These results suggest long-term hazard in use of drugs such as diazepam; they may be toxic and damage terminates in complex liver damage. Furthermore, melatonin and vitamin C may be useful in combating free radical-induced liver injury resulting from hazard and/or repeated diazepam administration.

INTRODUCTION

Benzodiazepines are the most frequently prescribed class of psychotropic drugs, possibly worldwide (Stiefel *et al.* 1999). Benzodiazepines, such as diazepam, are commonly used for their anxiolytic and sedative effects, that is, by their action on high-affinity receptor sites coupled to the γ -aminobutyric acid A receptor complex, present in the central nervous system (Costa *et al.* 1975). Nevertheless, in addition to the central receptors described for benzodiazepine, peripheral-type binding sites have been identified for them in liver cells (Woods *et al.* 1996), endocrine steroidogenic tissues (Zilz *et al.* 1999) and immune cells, such as macrophages and lymphocytes (Zavala 1997), and also in tumour cells (Hardwick *et al.* 1999; Han *et al.* 2003).

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Peripheral-type benzodiazepine receptor expression has been shown to increase in some neoplastic tissues and tumour cells, particularly in the liver (Venturini *et al.* 1999), ovary (Katz *et al.* 1990a), colon (Katz *et al.* 1990b), breast (Beinlich *et al.* 1999; Hardwick *et al.* 1999) and in the brain (Miettinen *et al.* 1995). Peripheral-type benzodiazepine receptor expression has also been associated with both tumour progression and aggressiveness, because higher levels of its expression were found in tumour cells that display increased rates of proliferation, such as some breast cancer cells (Hardwick *et al.* 1999; Corsi *et al.* 2005), hepatic cancer cells (Corsi *et al.* 2005) and glioma cells (Brown *et al.* 2000).

Many studies have been conducted to ascertain any role and/or involvement of free radical mediated pro-oxidative processes in the brain following diazepam administration (Musavi & Kakkar 1998, 2003). This team has reported that a single dose of diazepam caused stimulation of free radical-mediated changes. Melatonin can directly neutralize a number of free radicals and reactive oxygen and nitrogen species (Reiter *et al.* 2002) and stimulate several antioxidative enzymes such as superoxide dismutase and glutathione peroxidase (El-Sokkary *et al.* 2005). Free radical scavengers and antioxidants neutralize and/or metabolically remove reactive species from cells before they can carry out their destructive activities (Reiter *et al.* 2002). While the antioxidative actions of most molecules are limited by their specific intercellular distribution, antioxidative actions of melatonin include protection of lipids in cell membranes, proteins in cytosol and DNA in nuclei. Furthermore, melatonin can cross over all morphophysiological barriers and enter all cells in an organism (Reiter *et al.* 1999). Small molecule antioxidants, such as vitamin C (ascorbic acid) interact with oxidizing radicals (Jones *et al.* 1995). As an effective scavenger *in vivo* it can react with superoxide ions (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl ($\cdot OH$) ions, water-soluble peroxy radicals (RO_2), nitric oxide ($NO\cdot$), nitronium radicals (NO_2), singlet oxygen and hypochlorous acid (Halliwell 1994; Halliwell *et al.* 1995). From the above information concerning the role and action of diazepam on cell proliferation, DNA synthesis and oxidative stress, it seemed to be of interest to study the effect of repeated administration of diazepam, and beneficial roles of different antioxidants, on these parameters.

MATERIALS AND METHODS

Animals

Thirty-two adult male Sprague–Dawley rats, weighing approximately 125 g each, were used in this work. They were housed conventionally in cages, three to four rats per cage, and were fed with standard food and tap water, *ad libitum*. The rats were maintained in 12-h light:dark cycles at constant temperature of 25 ± 2 °C. An automatic timer controlled light and dark exposure with lights off 19:00 h to 07:00 h. Care and treatment of animals was approved and practices were performed according to approval of ethics regulation at the Assiut University.

Chemicals

Diazepam (Hoffman La Roche, Basel, Switzerland) and melatonin (Sigma Co., St. Louis, MO, USA) were suspended in a few drops of ethanol, and final volumes were adjusted with saline. 2-Thiobarbituric acid, 1,1,3,3-tetramethoxypropane, dimethyl sulphoxide, sodium dodecyl sulfate, superoxide dismutase (SOD), epinephrine and glutathione were purchased from Sigma. Tritiated thymidine ($[^3H]TdR$) was purchased from New England Nuclear (Boston, MA, USA). Kodak BTB₂ emulsion, Kodak D-76 developer and Kodak fixer were from Eastman Kodak (Rochester, NY, USA). All other chemicals were of the highest quality available.

Experimental design and procedures

Rats were divided into four equal-sized groups, eight rats per group. The first group served as controls and were dosed with vehicle solutions only. The second group was administered with diazepam (3 mg/kg) according to existing protocols (Musavi & Kakkar 2003). Animals of the third group were given a similar injection of diazepam that was preceded, 30 min earlier, by melatonin administration (5 mg/kg). The fourth group was given diazepam that was preceded, 30 min, by vitamin C (50 mg/kg) according to the technique of Siddique *et al.* (2005). All injections were subcutaneous, and were repeated daily for 30 days. At the end of the experiment, rats were killed and livers were removed and divided into two portions; one of them frozen and stored at -80°C and the other fixed in 10% neutral buffered formalin.

Autoradiography

Twenty-four hours after the last doses, all rats received a subcutaneous injection of one microcurie/gram body weight ($1\text{ }\mu\text{Ci/g}$ body weight) of $[^3\text{H}]\text{TdR}$ and they were killed 2 h later. Paraffin wax embedded sections ($5\text{ }\mu\text{m}$ thick) were prepared. Deparaffinized, they were dipped in Kodak NTB₂ emulsion (diluted 1 : 1 with distilled water) and were kept for 22 days in the dark at 4°C . All autoradiographs were stained with Harris's haematoxylin and eosin. A cell was scored as being labelled when it showed 5 or more silver grains over its nucleus.

Quantification of autoradiographs

A total of 12 000 labelled and unlabelled hepatocytes were counted in liver parenchymal areas. The ^3H -labelling index was expressed as percentage of labelled hepatocytes. Grain counts per labelled nucleus (GC/N) were evaluated by dividing the total number of silver grains over labelled nuclei by the total number of labelled cells. The labelling index and GC/N represent the kinetics of cell proliferation and rate of DNA synthesis, respectively (Hamatani *et al.* 1983), in these cells.

Measurement of lipid peroxidation

This method was based on that of Ohkawa *et al.* (1979) using 1,1,3,3-tetramethoxypropane as standard. A 10 (w/v) liver tissue homogenate was required for the assay, containing 1% v/v dimethyl sulphoxide to prevent further oxidation. To 0.2 mL aliquots of tissue homogenate was added 0.2 mL 8.1% w/v sodium dodecyl sulfate solution, 1.5 mL 20% v/v acetic acid solution (pH 3.5) and 1.5 mL 0.8% w/v thiobarbituric acid solution. Mixtures were made up to 4.0 mL with distilled water and were heated to 95°C for 1 h. Samples were cooled and were centrifuged at 2000 g for 10 min, and absorbance was measured at 532 nm. Results were expressed as n mol malondialdehyde formation per g tissue.

Superoxid dismutase activity

Superoxide dismutase activity was estimated based on its ability to inhibit the auto-oxidation of epinephrine in an alkaline medium (pH 10.2), according to Misra & Fridovich (1972); protein concentrations were measured by the method of Bradford (1976) using bovine albumin as standard.

Determination of glutathione

Glutathione was measured using the method of Beutler *et al.* (1963). This method is based on development of a stable yellow colour when 2-nitrobenzoic acid is added to sulphhydryl compounds.

Statistical analysis

Results were expressed as means $\pm$ SEM. Differences between means were tested by one-way ANOVA followed by the Student–Newman–Keuls *t*-test. The percent of stimulation (S%) or inhibition (I%) in the mean values was calculated.

Figure 1. Sections of rat livers showing distribution of labelled hepatocytes (arrows); and the density of grains over the labelled nuclei. (a) Control group. (b) Diazepam-administered group. (c) Diazepam + melatonin group. (d) Diazepam + vitamin C group. Haematoxylin and eosin ($\times 1000$ original magnification).

RESULTS

Cell proliferation in the normal liver parenchyma (hepatocytes) is rare, that is, the number of cells found in S-phase at any one time, is scarce. Accordingly, the ^3H -labelling index in this population is low. Distribution of labelled hepatocytes and the number of grains over labelled nuclei (GC/N) in the liver of control rats are shown in Fig. 1a. In rats that were administered with diazepam, the number of labelled hepatocytes and grains over labelled nuclei, were higher than those of controls, as shown in Fig. 1b. Quantitative results are presented in Fig. 2. There was a significant increase in mean values of labelling index and GC/N ($P < 0.01$) in diazepam-treated rats versus those of control animals, 135% and 127%, respectively. In rats that were given diazepam, preceded by melatonin or vitamin C administration, the numbers of labelled hepatocytes and the density of grains over labelled nuclei were lower than those of diazepam-treated rats

Figure 2. Mean ³H-labelling indices and grain counts, per labelled nucleus in hepatocytes of control and the three treated groups of rats. Lettered groups indicate significant differences, identical lettering no significant differences. Data are means ± SEMs.

Table 1. Mean values ± SEM of lipid peroxidation product (MDA), superoxide dismutase (SOD), glutathione (GSH) and inhibition (I%) and/or stimulation (S%) in the liver homogenates of control and different treated groups of rats

Groups	Measurements		
	MDA (nmol/g tissue)	SOD (Units/mg protein)	GSH µg/mg protein
Control	4.298 ± 0.256 ^a	1.132 ± 0.065 ^a	0.0083 ± 0.00024 ^a
Diazepam	9.276 ± 0.204 ^b	0.427 ± 0.037 ^b	0.0047 ± 0.00015 ^b
I or S% versus control	S = 115.8	I = 62.3	I = 43.6
Diazepam + melatonin	4.871 ± 0.290 ^{ac}	0.691 ± 0.035 ^c	0.0063 ± 0.00013 ^c
I or S% versus diazepam	I = 47.4	S = 61.8	S = 35.3
Diazepam + vitamin C	5.357 ± 0.104 ^c	0.737 ± 0.046 ^c	0.0065 ± 0.00022 ^c
I or S% versus diazepam	I = 42.2	S = 72.6	S = 40.6

SEM, standard error of the mean. Values in the same columns with unlike superscript letters significantly differ.

(Fig. 1c,d, respectively). Reduction in labelling index and GC/N were significant ($P < 0.01$), 44% and 28%, respectively, in the melatonin group while being 36% and 25% in the vitamin C group. There was no significant difference between melatonin- and vitamin C-administered groups.

In diazepam-treated rats, there were measured increases in lipid peroxidation (LPO) levels (as indicated by higher MDA) in liver versus controls (Table 1). Statistically, the increase was significant ($P < 0.01$) at around 116%. When melatonin or vitamin C was provided to diazepam-treated rats, levels of LPO were lower. The statistical analysis of these data indicated that the reduction was significant ($P < 0.01$), 47% and 42% for melatonin and vitamin C, respectively,

versus those of diazepam-treated rats (Table 1). Melatonin exerted a higher inhibitory effect than vitamin C in the case of LPO.

Superoxid dismutase (SOD) activity was inhibited significantly ($P < 0.01$) in livers of diazepam-treated rats versus those of controls (Table 1). Melatonin or vitamin C administration to diazepam-treated rats significantly stimulated ($P < 0.01$) SOD activity by 62% and 72%, respectively. Vitamin C exerted a higher stimulatory effect than melatonin in the case of SOD expression.

Diazepam administration significantly reduced ($P < 0.01$) glutathione levels in the liver compared to those of controls by 44% (Table 1). When melatonin or vitamin C was given to diazepam-treated rats, they significantly stimulated ($P < 0.01$) levels of glutathione by 35% and 40%, respectively. Again, vitamin C exerted a higher stimulatory effect than melatonin in the case of glutathione production.

DISCUSSION

Diazepam, the active ingredient of Valium^{RT}, belongs to the class of drugs known as benzodiazepines. These drugs affect the central nervous system and are commonly prescribed as sedatives, hypnotics, anticonvulsants and skeletal muscle relaxants (Baum *et al.* 1984). It has been pointed out that some benzodiazepines, such as diazepam, bind to both central and peripheral benzodiazepine receptors with high affinity (Pawlikowski *et al.* 1988a; Gorman *et al.* 1989). The role of the peripheral-type receptors seems to be connected with more slowly appearing drug action, such as modulation of cell proliferation (Pawlikowski *et al.* 1988a; Saano 1988). It has been reported that diazepam (a peripheral-type benzodiazepine receptor ligand) induces free radical-mediated changes (Musavi & Kakkar 2003).

In the current study, diazepam administration increased the levels of LPO and decreased activity of SOD and glutathione (GSH) levels (parameters of oxidative damage) in rat livers. Musavi & Kakkar (1998) reported an involvement of free radical-mediated pro-oxidative processes in brain following diazepam administration. They found enhancement in thiobarbituric acid-reactive substance formation by 107% compared to controls. Furthermore, they found decreases in SOD and GSH reductase activity. Elevated oxidized LPO, as an offensive system and lowered SOD activity and GSH levels, as a defence system, played an important role in development of oxidative stress in livers of diazepam-treated rats in this study.

Results obtained here reveal that administration of antioxidants (melatonin and vitamin C) significantly decreases tissue LPO and increase tissue SOD activity and GSH levels. It was also found that melatonin exerts a more inhibitory effect for LPO and less stimulation for SOD and GSH, than vitamin C. It has been suggested that melatonin was one of the first biological signals to appear, in an evolutionary context (Claustrat *et al.* 2005). Various studies have supported the value of exogenous administration of melatonin in oxidative damage (El-Sokkary 2000, 2002, 2007; Karasek *et al.* 2002).

The antioxidative protection by melatonin includes direct scavenging of free radicals, and electron exchange reactions of the mitochondrial respiratory chain (Pandi-Perumal *et al.* 2006). Melatonin antioxidant capacity also includes the indirect effect of up-regulating several antioxidative enzymes and down-regulating pro-oxidant enzymes (Zhang *et al.* 1999), and nitric oxide synthases (Storr *et al.* 2002). Direct and indirect antioxidative functions of melatonin may explain decrease of LPO end product (MDA) and increase of SOD activity and GSH levels.

Vitamin C plays an important physiological role in cells as a reducing agent, antioxidant, free radical scavenger and enzyme cofactor (Padayatty *et al.* 2003). Vitamin C and glutathione show strong functional interdependence *in vivo* (Wu *et al.* 2004). Meister (1994) reported that vitamin C supplementation ameliorated dysfunction and mortality associated with glutathione deficiency. Interestingly, when GSH deficient animals were supplemented with vitamin C, beside the expected rise in tissue ascorbate levels, there was also a remarkable increase in cellular glutathione (Martensson & Meister 1991). These results may explain the increment of GSH levels in rats administered with vitamin C over those given melatonin. It has been reported that vitamin C increases the activity of testicular SOD in rats (Gupta *et al.* 2004).

In the current study, diazepam administration exerted stimulatory action on cell proliferation and ^3H -thymidine incorporation during S phase (DNA synthesis) in hepatocytes of treated rats. These results are in agreement with those of other studies that found that diazepam injection into male rats resulted in a significant increase in the proliferative activity of thymic cells (Stepien *et al.* 1988) and increased mitotic activity in cells of the anterior pituitary (Pawlikowski *et al.* 1987). Contrary to these results, Pawlikowski *et al.* (1986) described a specific dose-related inhibitory effect of diazepam on spontaneous proliferation of mouse spleen lymphocytes *in vitro* and suppressed ^3H -thymidine incorporation into DNA of human glioma cells *in vitro* (Pawlikowski *et al.* 1988b). A recent study of Sakai *et al.* (2006) reported positive staining of peripheral-type benzodiazepine receptors in Ehrlich tumour cells, and direct effects of diazepam on Ehrlich tumour cell proliferation. Particularly, the peripheral-type benzodiazepine receptor ligand, diazepam, decreased the percentage of cells in G_0/G_1 phase, and increased the percentage of cells in S and G_2/M phases. Effects of diazepam on Ehrlich tumour cells are specifically derived from peripheral-type receptor activation (Sakai *et al.* 2006). The increase in hepatocyte proliferation and ^3H -thymidine incorporation observed in the current study after repeated diazepam administration, might be taken as a consequence of a direct effect of this drug on receptors expressed in hepatocytes and/or is due to oxidative stress induction.

Here, administration of the antioxidants (melatonin and vitamin C) attenuated proliferative activity and ^3H -thymidine incorporation (DNA synthesis) in hepatocytes of rats given diazepam. Furthermore, these results indicate that melatonin presented a more inhibitory effect on previous parameters than vitamin C. It was found that melatonin inhibited cell proliferation in several tumour types (Hill & Blask 1988) and in several other systems (Blask 1993; El-Sokkary 2002). Melatonin is also an inhibitor of cell cycle progression, thereby increasing the number of cells in G_1 phase and reducing the percentage of cells in S phase (Cos *et al.* 1991; El-Sokkary *et al.* 2003; El-Sokkary 2007). At least one of the mechanisms implicated in the antiproliferative effect of melatonin is its action as an antioxidant, because it prevents decrease in mRNA of antioxidant enzymes induced by diazepam.

Vitamin C has been found to inhibit tumour cell growth, binding active carcinogenic metabolites to cell DNA (Chen *et al.* 1988). Osmak *et al.* (1997) also reported an inhibitory effect of vitamin C on some tumour cell growth *in vitro* and *in vivo*; in addition, vitamin C has potential anticancer activity; but its possible role and mechanism of action in cancer prevention has not yet been clearly established.

In the light of these results, it can be suggested that diazepam is an oxidative stress inducer. Melatonin is better than vitamin C in providing effective reduction of diazepam-induced liver cell proliferation, DNA synthesis and LPO; while vitamin C is better than melatonin in providing stimulation of SOD activity and GSH levels. Thus, administration of antioxidants could be recommended for effective treatment and may find clinical application against a variety of drug hazards where cell damage is a consequence of free radicals activity.

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