Protective role of melatonin on the effect of diazepam on proliferative activity, morphological changes and testosterone levels in the testes of rats

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Abstract

Objective: There are evidences indicating that diazepam administration inhibits cell proliferation both in vitro and in vivo. The benefits of melatonin against diazepam-induced proliferative activity, morphological and level of testosterone changes were investigated in this study.

Materials & methods: Three equal-sized groups of male rats [control, diazepam (3 mg/kg) and diazepam plus melatonin (10 mg/kg)] were used. Levels of testosterone hormone in the serum were measured. Cell proliferation was detected by autoradiography and the morphological alterations were investigated.

Results: Results documented decreased both 3H-labelling index (proliferative activity) and the levels of testosterone with marked histopathological alterations in the seminiferous tubules of diazepam-administered rats versus those of controls. When melatonin was given to diazepam-administered rats, it stimulates the decrease of labelling index, restored the levels of testosterone and preserves the alterations and number of different germ cell types in all stages of the seminiferous tubules.

Conclusion: These results suggest long-term hazard in use of drugs such as diazepam may be toxic and damage terminates in complex testes dysfunction and infertility. Furthermore, melatonin may be useful in combating testes injury resulting from hazard and/or repeated diazepam administration.

Keywords: Melatonin, diazepam, 3H-labelling index, seminiferous tubule

Introduction

Benzodiazepines (BDZ) are the most frequently prescribed class of psychotropic drugs may be worldwide [1]. Benzodiazepines, such as diazepam, are commonly used for their sedative and anxiolytic effects, i.e., by their action on high affinity receptor sites coupled to gammaaminobutyric acid A (GABA-A) receptor complex, present in the central nervous system (CNS) [2]. In addition to the central receptors described for BDZ, peripheral-type binding sites (PBRs) have been identified for them in liver cells [3], endocrine steroidogenic tissues [4] and immune cells, such as macrophages and lymphocytes [5], and also in tumor cells [6,7].

Peripheral-type benzodiazepine receptor expression has also been associated with both tumor progression and aggressiveness, since higher levels of its expression were found in tumor cells that display increased rate of proliferation, such as breast cancer cells [6], hepatic cancer cells [8] and glioma cells [9].

There are many evidences indicating that diazepam inhibits cell proliferation both in vitro and in vivo. Clark and Ryan [10] reported that diazepam exerted an antiproliferative effect on 3T3 fibroblasts. The antiproliferative action of diazepam on the proliferation of mouse spleen lymphocytes in vitro was noticed by Pawlikowski et al. [11,12]. Also, it was found that diazepam inhibited the in vitro proliferation of rat C6 glioma and mouse neuroblastoma cells [13] and cerebral cortical neurons [14]. In vivo studies of Pawlikowski et al. [15] showed that diazepam decreased the mitotic activity of the parietal cerebral cortex and the anterior pituitary gland in rats. Diazepam administration...
was shown to inhibit the mitotic index of the adrenocortical cells [16] and suppress the proliferation of spleen lymphocytes in rats [17]. In contrast, El-Sokkary [18] found increased labelling index and [3H]-thymidine incorporation (DNA synthesis) in the liver of rats administered with diazepam. Diazepam had antifertility effects on male rats through attenuating steroidogenesis and testosterone production by inhibiting the pituitary gonadal axis hormones and the StAR gene expression through its effects on calcium ions [19].

Melatonin, a hormone which is synthesized by the pineal gland, is a potent antioxidant [20, 21]. Although melatonin altered the spermatogenesis, it could not change the morphometric parameters such as tubular diameter and heights of the epithelium significantly [22]. Melatonin, by having lipophilic and hydrophilic compounds, can pass freely from the morpho-physiological barrier of different organs [23]. The direct effect of melatonin was observed in the male reproductive system and in the synthesis of testosterone from the Leydig cells in animals [24].

In general, it is accepted that a higher percentage of motile sperm is associated with improved fertilization rates and Ortiz et al. has shown that the addition of melatonin to seminal samples can improve the overall motility and the percentage of progressively motile spermatozoa [25, 26]. Melatonin also appears to inhibit apoptosis in spermatozoa, with a reduction in early apoptotic events being demonstrated in human sperm thus prolonging sperm survival [27]. These effects would serve to improve sperm quality, therefore increasing the probability of successful fertilization. Melatonin, through its neutralization of reactive oxygen and nitrogen species, has been shown in both animal and human studies to improve seminal quality in-vitro. Studies in rats have shown that melatonin has a positive effect on sperm that have been subjected to oxidative stress, improving sperm number, viability and motility [28, 29]. Similar results have been found in a small human study in which in-vitro melatonin-treated samples showed a higher percentage of sperm motility and a lower proportion of non-viable spermatozoa [30].

Materials and Methods

Animals

Thirty adult male Sprague-Dawley rats, weighing approximately 150 g, were used in this work. The animals were housed conventionally in cages (3-4 rats per cage) and fed with standard food and tap water ad libitum. The rats were maintained in 12:12 light:dark cycle at constant temperature (25±2°C). An automatic timer controlled light and dark exposure with lights off 19.00h to 07.00h. The care and treatment of the animals was approved and performed according to the approval ethics of Assiut University.

Chemicals

Diazepam (Hoffman La Roche, Basel, Switzerland) and melatonin (Sigma Co. St. Louis MO) were suspended in few drops of ethanol and the final volumes adjusted with saline. Tritiated thymidine ([3H]Tdr) was purchased from PerkinElmer (Boston, MA). Kodak NBT2 emulsion, Kodak D-76 developer and Kodak fixer were from Eastman Kodak (Rochester, New York). Fertigenix-Testo-Easia kit (Biosource, USA). All other chemicals were of higher quality available.

Experimental Design and procedures

The rats were divided into three equal-sized groups 10 rats each. The first group served as control and given vehicle (0.9% NaCl solution contained 0.5% ethanol) only. The second group was administered with diazepam (3 mg/kg) according to El-Sokkary [18]. The third group was given a similar injection of diazepam which was preceded, 30 min earlier, by melatonin administration (10 mg/kg) according to El-Sokkary et al. [20]. All of these injections were subcutaneously repeated daily for 30 days. At the end of the experiment, the rats were sacrificed and the testes were removed and fixed in 10% neutral buffered formalin.

Autoradiography and quantitation of autoradiographs

Twenty four hours after the last injections, 5 rats from each group received a subcutaneous injection of 1 uCi/g body weight of [3H]Tdr and they were killed 2 hours later. Paraffin sections (5 mm thick) were prepared. The deparaffinized sections were dipped in Kodak NTB2 emulsion (diluted 1:1 with distilled water) and kept for 25 days in the dark at 4°C. All autoradiographs were stained with Harris’s haematoxylin and eosin. A spermatogonial cells were scored as being labelled when 5 or more grains located over their nuclei. All labelled and unlabelled spermatogonia in the 8 stages of the seminiferous tubes were counted. The [3H]-labelling index (LI) was expressed as the percentage of labelled spermatozoa. The LI represent the kinetics of cellular proliferation [31].

Measurement of serum testosterone

Serum samples were collected for testosterone determination using ELISA. Levels were measured by using the above kit according to the manufacturer’s instructions.

Histology and quantitation of seminiferous epithelial cycle and Leydig cells

5 rats from each group were sacrificed concomitantly with the previous rats and specimens of testes were taken, fixed, embedded in paraffin, sectioned and stained with haematoxylin and eosin. The identification of the different stages of seminiferous epithelial cycle (50 seminiferous tubules for each animal) was based on the morphological changes of the germ cell nuclei and the local arrangement of the spermatids [32, 33].

The number of Leydig cells/mm² (unit area) was counted using the square grid (area/mm²) at 1000X magnification and 35 unit areas were counted. The nuclear volume of the Leydig cells was performed using ocular micrometer at 1000X magnification. A total number of 300 nuclei were measured/animal.
The measurement was carried out by concerning rounded nuclei of Leydig cells where the formula of the sphere was applied \( V = \frac{4}{3}\pi R^3 \), where \( R = \text{nuclear diameter}/2 \).

**Statistical analysis**
Quantitative results were expressed as means±S.E.M. Differences between means were tested by one way analysis of variance (ANOVA) followed by the Student-Newman-Keuls t-test. The percent of stimulation (S%) or inhibition (I%) in the mean values was calculated.

**Results**

**The seminiferous tubules**
In the diazepam-treated rats, the diameters of seminiferous tubules decreased and their architecture was altered (Figures 2 and 5) when compared with those of control animals (Figures 1 and 4). A lot of degenerating germ cells that contain vacuoles of varying sizes, congested blood vessels and peritubular edema were detected in large number of seminiferous tubules of diazepam-treated rats as shown in Figures 2 and 5. Also, the number of germ cells in all stages of the seminiferous tubule markedly decreased in diazepam-treated rats compared to those of controls (Figure 5). Melatonin administration to diazepam-treated rats restored the changes of the germ cells towards those of controls (Figures 3 and 6).

The quantitative results of the seminiferous tubules cycles are presented in Table 1. Statistically, there was a significant reduction \( P<0.01 \) in the mean number of different germ cell types in all stages of the seminiferous tubules in diazepam-treated rats versus those of control animals. When melatonin was given to diazepam-treated animals, the number of different germ cell types in all stages of the seminiferous tubules was significantly increased \( P<0.01 \) and appeared near to those of control numbers.

**The interstitial cells of Leydig**
As seen in Figure 2 there was a sharp drop in the number of Leydig cells in the testes of diazepam-treated rats when compared with those of controls (Figure 1). The quantitative results of the number and nuclear volume of Leydig cells
are presented in Table 2. As shown, there was a significant decrease (P<0.01) in the mean number of Leydig cells/mm (unit area) in diazepam-treated rats versus controls. Also, the quantitative data revealed that there was a significant reduction (P<0.01) in the mean volume of nuclei of Leydig cells in diazepam-treated rats versus controls. Again, melatonin administration to diazepam-treated animals increased both of the mean number of Leydig cells and the mean volume of their nuclei. The stimulation in the two parameters was significant (P<0.01).

**Testosterone concentration**

Serum testosterone concentrations were significantly decreased (P<0.01) by 36% in diazepam-treated animals versus controls (Table 3). When melatonin was administered to diazepam-treated rats, it stimulated the concentration of testosterone hormone by 18.6%. Statistically this stimulation was significant (P<0.05).

**Autoradiography and spermatogonial proliferation**

The number of labelled spermatogonia in the different stages of seminiferous tubule cycle was lower in diazepam-treated rats than those of controls, as shown in Figures 7 and 8. The quantitative results of autoradiographic study are presented in Table 4. As shown, the mean ^3H-labelling index (LI) was significant decreased (P<0.01) in spermatogonia of each stage in the seminiferous tubule cycle of diazepam-treated rats versus those of controls. In addition, the mean value of LI in the spermatogonia of all stages of the seminiferous tubule cycle as a whole decreased significantly (P<0.01) in rats given diazepam versus control ones. The above data revealed that there was a sharp suppression in the proliferative activity of spermatogonia in diazepam-treated rats. However, it was restored upon melatonin administration to these rats.

**Table 1: Mean number of cells (±S.E) in different stages of the seminiferous epithelial cycle in control and treated rats.**

| Stages | Sertoli cells | Spermatogonia Type A | Spermatogonia Type B | Total | Primary Spermatocytes | Secondary Spermatocytes | Spermatids | Rounded | Elongated | Spermatzoa |
|--------|---------------|----------------------|----------------------|-------|-----------------------|-------------------------|------------|---------|----------|-----------|
| Control group | 4.64±0.171 | 28.7±0.981 | 8.2±0.181 | 36.9±0.932 | 52.94±1.194 | 7.84±0.182 | 49.3±1.568 | 9.86±0.293 | 12.28±0.316 |
| DZ group  | 2.52±0.162  | 17.8±0.762  | 4.7±0.162  | 22.5±0.814 | 32.58±1.057 | 3.24±0.101 | 32.58±1.057 | 8.3±0.179  | 9.1±0.222  |
| DZ+melatonin | 3.68±0.165 | 22.3±0.684 | 6.9±0.244 | 29.1±0.738 | 43.6±1.298 | 5.42±0.172 | 43.6±1.298 | 9.28±0.252 | 10.98±0.207 |

Values in the same column with unlike superscript letters are significantly differing at P<0.05.

**Table 2: Means of number and nuclear volume of Leydig cells in control and treated rats.**

| Measurements | Mean number of Leydig cells±SD | Mean nuclear volume (mm³) of Leydig cells ±SD |
|--------------|--------------------------------|---------------------------------------------|
| Control group | 24.8±4.3³ | 89.6±8.2² |
| DZ group | 12.2±2.3³ | 43.5±5.2² |
| DZ+melatonin | 17.6±1.9³ | 60.7±4.3³ |

Values in the same column with unlike superscript letters are significantly differing at P<0.05.
Table 3: Means of testosterone concentrations (± SE), inhibition (I%) and/or stimulation (S%) in control and treated rats.

| Measurements          | Animal group | Control       | DZ group      | DZ+melatonin |
|-----------------------|--------------|---------------|---------------|--------------|
| Testosterone (ng/ml)  |              | 0.857±0.02a   | 0.548±0.02b   | 0.673±0.02c  |
| I % versus Cont.      |              | I= 36.0       | I= 21.5       | S = 18.5     |

Values in the same row with unlike superscript letters are significantly differing at P<0.05.

Table 4: Mean 3H-labelling indices (± SE) in spermatogonia of all stages of seminiferous tubules in control and treated rats.

| Stages | Groups      | I    | II   | III  | IV   | V    | VI   | VII  | VIII  | Mean  |
|--------|-------------|-----|------|------|------|------|------|------|-------|-------|
|        | Control group | 17.1±0.037 | 9.9±0.40 | 11.8±0.38 | 17.7±0.36 | 12.2±0.37 | 21.4±0.43 | 18.2±0.34 | 11.2±0.33 | 14.9±1.47 |
|        | DZ group     | 8.25±0.20 | 6.45±0.25 | 7.1±0.27 | 8.56±0.22 | 6.5±0.28 | 9.15±0.21 | 8.54±1.05 | 6.4±0.29 | 7.62±0.40 |
|        | DZ+melatonin | 12.95±0.27 | 8.05±0.22 | 8.9±0.24 | 11.35±0.38 | 8.55±0.26 | 15.4±0.47 | 13.85±0.28 | 10.25±0.31 | 11.16±0.95 |

Values in the same row with unlike superscript letters are significantly differing at P<0.05.
ACTH secretion; accordingly, the secretion of adrenocortical hormones decreased [35]. The inhibitory effect of diazepam on cell proliferation may due to the induction of chromosomal malsegregation. It was found that diazepam inhibit centrioles shifting and induces the formation of monopolar spindle during the metaphase-anaphase transition [36].

Another possibility, which does not exclude the preceding mechanisms, is the effect of diazepam on calcium ions. It was reported that BZD receptors block the voltage-dependent calcium channels [37]. It is well stablished that calcium ions are needed in the later G1 phase of the cell cycle for the initiation of DNA synthesis [38]. Moreover, it was reported that BZD inhibits the calcmodulin-dependent protein kinase in the brain. Jones et al. [39] found that the role of calcium in the initiation of DNA synthesis is mediated by the formation of calcium –calcmodulin complex.

Our results showed that melatonin administration enhances the proliferative activity of the spermatogonia and the number of labelled cells increased matching those of controls. The fascinating feature of this hormone is that it makes the normal cells proliferate and makes the cancerous cells undergo apoptosis [40].

Increasing evidence demonstrates that melatonin has an anti-apoptotic effect [41-43]. In vivo, melatonin alleviated ischemia-reperfusion induced apoptosis in neural cells through repression of the mitochondrial permeability transition pore [44,45]. Wang et al. [46] found that melatonin attenuated lipopolysaccharide-induced apoptotic liver damage in mice [46]. Li et al. [47] reported that melatonin protected kidney grafts from ischemia/reperfusion injury through the repression of the apoptosis after experimental kidney transplantation. In the study of Ji et al. [48], they found that melatonin alleviated Cd-induced testicular histopathological damage. Importantly, the frequency of tubules with more than six apoptotic germ cells was significantly reduced when mice were administered with melatonin. In addition, melatonin significantly reduced the percentage of tubules with apoptotic germ cells and the number of apoptotic germ cells per tubule. Also, melatonin attenuated 2-bromopropane-induced testicular germ cell apoptosis in rats [49]. These data demonstrate that melatonin could protect against germ cell apoptosis in testes.

In the current study, degeneration in the cells of the seminiferous tubules, vacuolation in their cytoplasm, and reduced seminiferous epithelial layers, edema, and hyalinization of intertubular tissue were detected in diazepam treated animals. The same results was reported previously by other investigators [50,51], who described the appearance of necrosis, degeneration, desquamation, disorganization, reduction in germinal cells, vacuolization in Sertoli cells, multinucleated giant cell formation, interstitial edema, and congestion in the intertubular tissue.

In the present study, the concomitant administration of melatonin with diazepam led to a marked improvement in the histopathological changes. These changes were in agreement with those observed by some researchers [52-54], who reported that melatonin may alleviate cadmium-induced cellular stress and germ cell apoptosis in testes.

Moreover, Take et al. [55] evaluated the protective effect of melatonin against irradiation-induced damage to rat testes that included amelioration of germ-cell depletion and apoptotic changes. At the ultrastructural level, Hussein et al. [56] observed the disappearance of the characteristics of apoptosis (condensation of the nuclei, vacuolization of the cytoplasm, increased cytoplasmic density, and apoptotic bodies) when the irradiated animals were pretreated with melatonin.

A lot of studies have explained the possible mechanism of melatonin protection as being dependent on its antioxidative action. Lena and Subramanian [57] reported that melatonin has the ability to scavenge reactive species, which makes melatonin a potent antioxidant and a free radical scavenger. They concluded that melatonin could control the oxidative abuse by (i) directly scavenging a variety of radicals and ROS, (ii) inducing antioxidative enzymes which reduce the steady-state levels of ROS, (iii) inhibiting nitric oxide synthase, which generates nitric oxide, and (iv) stabilizing cell membranes that aid them in reducing oxidative damage.

Gutierrez-Cuesta et al. [58] reported that melatonin improved prosurvival signals and reduced pro-death signals. Zhang et al. [59] have suggested that melatonin may potentially attenuate testicular damage by improving morphological changes and reducing germ cell apoptosis in mice.

The development and growth of male reproductive ducts and seminiferous tubules is dependent on the increase of testosterone concentration [60]. Testosterone is essential for the survival of the spermatogenic endothelium and the significant decrease in testosterone hormone causes an increase in sperms abnormal morphology and decrease in sperm viability [61].

The results of the present study had shown that treatment with diazepam led to significant decrease in testosterone level in serum of treated animals. Testosterone hormone is the principle male hormone; it is synthesized by Leydig cells from cholesterol [62]. This decrement of testosterone level may be due to the effect of diazepam on serum cholesterol which is a precursor of testosterone synthesis by its action on the Leydig cells [63]. Therefore, the decreased testosterone concentration produced by the drug could be explained either by direct effect at Leydig cell level or an indirect effect by disturbing the hormonal milieu at hypothalamic-pituitary axis [64].

The reduced level of the testosterone has been suggested as being caused by the oxidative damage to the Leydig cell population [65] and might be related to the decreased numbers of Leydig cells [66]. Melatonin is attracting increased attention in recent years due to its known ability to reduce oxidative stress [20], with negligible toxicity even in very high doses [67-69]. Melatonin is not only an effective hydroxyl radical
Melatonin treatment to diazepam-administered rats ameliorated the reduction in the testosterone level. The increase of testosterone level after melatonin administration to diazepam-treated rats lends credence to melatonin's protection against the primary causes of damage of the testis. Because testosterone plays a key role in the process of spermatocyte meiosis and the relationship between melatonin and testosterone has been well documented, this modulation of the testosterone level by this hormone potentially protected the meiosis of spermatocytes and production of normal sperm quality, and this effect might be attributed to its capacity to control cellular redox state.

Finally, in this study, it was found that melatonin could provide good protection against diazepam-induced testicular damage. Several clinical trials should be conducted in the future to combine melatonin treatment with other chemotherapeutic and toxic drugs to reduce their cellular damage and toxicity.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions

| Authors' contributions                              | GHS | HHR | HAY |
|-----------------------------------------------------|-----|-----|-----|
| Research concept and design                         | ✓   | ✓   | ✓   |
| Collection and/or assembly of data                  | ✓   | ✓   | ✓   |
| Data analysis and interpretation                    | ✓   | ✓   | ✓   |
| Writing the article                                  | ✓   | ✓   | ✓   |
| Critical revision of the article                    | ✓   | ✓   | ✓   |
| Final approval of article                           | ✓   | --  | --  |
| Statistical analysis                                | ✓   | --  | --  |

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