

Immunotoxicity of Clonazepam in Adult Albino Rats

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Clonazepam as an addictive drug is studied to elucidate its destructive effects on rats' immune system. The aim of the current work was to study the immunologic changes induced by sub-chronic administration of clonazepam for three weeks followed by a withdrawal period in adult male albino rats. Seventy-two Sprague Dawley rats were divided into three equal groups. The first group was used as control; the second and third groups were treated with clonazepam. Six rats from each group were sacrificed weekly. Data showed that clonazepam induced a significant suppression in the level of IFN- γ , cortisol production, total splenocytes count and lymphocytes transformation induced by PHA mitogen along the experimental period especially in the third group. However, subchronic doses of clonazepam increased the production of IL-10 in both treated groups. Moreover, significant DNA damage in the peripheral blood lymphocytes of both treated groups was observed along the duration of the study. In conclusion, the immune system responses can be adversely affected to a greater extent by sub-chronic administration of clonazepam and should be prescribed cautiously as patients may turn addict to it.

Many pharmaceutical substances may lead to addiction; furthermore, a number of such drugs are mismanaged as well as abused (Koob & Le-Moal, 2001; Charlson *et al.*, 2009; Berger *et al.*, 2012). Among the drugs that are prescribed for medicinal purposes include barbiturates, benzodiazepines (BZD), cough sedatives and narcotic analgesics. Also patients may also turn to be drug abusers, whereas drug dealers take advantage of their situation by the distributing such known addictive drugs (Lance & Brian, 2000; Uzun *et al.*, 2010).

The benzodiazepines form one of the largest classes of abused pharmaceuticals. The drugs in this class are numerous and are included under Schedule IV control (Allan *et al.*, 1992). Benzodiazepines are used clinically as tranquilizers, muscle relaxants, anticonvulsants, anxiolytics, and sedative-hypnotics. These effects are mediated primarily via the central benzodiazepine receptors (CBR) located in the CNS (Heiss & Herholz, 2006). However, it has been found that BZD also act on peripheral benzodiazepine receptor sites (PBR), an

18kDa translocator protein (TSPO) (Papadopoulos *et al.*, 2006). Several reports identified BZP peripheral type binding sites in endocrine steroidogenic tissues, organs and cells of the immune system, such as macrophages and lymphocytes. Thus, the PBR may be a possible primary target for the immunotoxic effects of BDZ (Righi *et al.*, 1999). A recent study demonstrated that a direct immunomodulatory action for BZD due to the presence of TSPO on immune/inflammatory cells (De Lima *et al.*, 2010): Clonazepam, a recently developed benzodiazepine derivative is nowadays widely prescribed for a variety of psychiatric and neurologic conditions (Morishita, 2009). It has been found that it is also frequently abused as a street drug and is a prevalent illicit drug among addicts in most of the new world countries (Heberlein *et al.*, 2009). Clonazepam is a high-potency benzodiazepine with a long half-life, so symptoms of withdrawal may not begin for several days after the drug is discontinued (Juergens, 1993). Sudden withdrawal after long-term administration may lead to dysphoria, restlessness, irritability, sleepiness, hand

tremors, oral dyskinesias, muscle rigidity and seizures (Heberlein *et al.*, 2009).

One of the most important factors in our understanding of the regulation of immune responses was the description T helper 1(Th1)/T helper 2 (Th2) paradigms. Th1 cytokines, are for instance interferon- γ (IFN- γ), interleukin (IL)-2, and generally promote cellular immune responses, whereas the Th2 cytokines, IL-4, IL-5, IL-9, IL-10 and IL-13, provide optimal help for humoral immune responses (Mosmann *et al.*, 1986). In general, type 1 and type 2 cytokines are reciprocally regulated; IFN- γ inhibits the proliferation of Th2 cells, whereas IL-10 inhibits that of Th1 cells (Swain *et al.*, 1991). IFN- α , acts by supplement to control cellular immunity mainly by promoting the effector functions of lymphocytes and activating monocytes and macrophages, while it also has an antiviral activity.

On reviewing the available literature from a toxicological point of view, we noted limited information about the influence of clonazepam on the immune system. Among methods used for the evaluation of cytotoxicity of chemicals, the measurement of apoptosis becomes the essential component, through knowing its toxic mechanism (Sperandio *et al.*, 2000). The aim of the current work was to study the differential immunologic changes induced by sub-chronic administration of clonazepam at their maximum therapeutic doses following a withdrawal period by measuring Th1/Th2 cytokines, serum cortisol level and DNA degradation on peripheral blood lymphocytes of adult male albino rats.

Materials and Methods

Animals

Adult males Sprague Dawley albino rats were used in this study; all were with an average weight of 175gm. Animals were supplied by the Theodore Billharz Research Institute (TBRI) animals house. The rats were

individually caged in a colony room, where a reversed day-night (12 hr) cycle was maintained through artificial illumination. Rats received free access to both food pellets (protein 21%, energy 2950 Kcal. and water throughout the experiments. Animals were kept for a week acclimating before beginning the study.

Rats (n=72) were randomly assigned to three equals groups, the first group (grp 1) was used as controls and received 0.1 ml distilled water throughout study period. The second (grp2) and third (grp 3) groups were treated with daily doses of Amotile (clonazepam tablets), produced by Amoun pharmaceutical Co., El-obour City, Cairo, Egypt. Dependence to clonazepam was induced by starting with the therapeutic dose for rats according to conversion tables, considering that therapeutic dose for human 4mg/day (Paget and Barnes 1964; Martindale 2008). The starting clonazepam oral dose for rats in grp2 and grp 3 was 0.36 and 1.08 mg/kg respectively. These daily doses of clonazepam were doubled weekly in grp2 and grp3 to reach 1.08 and 4.32 mg/kg respectively. At the end of the three weeks treatment, rats were then kept for one more week before starting to evaluate the different immunological parameters influenced by drug abstinence; six animals from each group were sacrificed weekly.

Determination of serum IFN- γ and IL-10

To investigate the effect of clonazepam administration on T-helper (Th1 and Th2) cell function, we determined levels of IFN- γ (Th1 cytokine)/ IL-10 (Th2 cytokine) using two different immunoassay kits {Abnova (cat # KA0274.V.01, Taiwan) and PBL Biomedical Laboratories (product # 43500-1, V.1.3, Taiwan) respectively, according to manufacturer's instructions.

Cortisol level

The Calbiotech, Inc. cortisol ELISA testing kit (Catalog No. NC0366074, USA) was used to quantitative measurement of cortisol in serum or plasma, according to manufacturer's instructions.

Splenocytes preparation

Study rats' spleen were individually removed and placed in cold culture media (RPMI-1640) containing 100U/ml penicillin, 100 μ g/ml, streptomycin, 200mM Glutamine/100 mM sodium pyruvate. Each spleen was gently teased loose and passed through a stainless steel mesh (40 μ m pores) to remove cell aggregates and connective tissue. Each suspension was centrifuged at + 4°C for 10 min at 400g. The pellet was suspended in 1ml sterile distilled water for 30 sec to lysis the red blood cells, then 40 ml RPMI medium were

immediately added followed by centrifugation at 400g at +4°C for 5 min. Cells were washed twice with ice-cold RPMI-1640. Cells count was determined and viability assessed using trypan blue (Fluka Chemie AG CH-9470, Switzerland).

Lymphocytes transformation

MTT (3,4,5-dimethylthiazoyl-2,5-diphenyl tetrazolium bromide) colorimetric analysis was used to measure the proliferation rate of lymphocytes. The effect of the drug on lymphocyte transformation was performed according to Mosmann, (1983). Briefly, heparinized blood samples were obtained from all study rat groups layered carefully on the surface of Ficoll-hypaque solution (Pharmacia Biotech, Winthrop Pharmaceuticals, New York.) at a ratio of 2/1 and centrifuged (Sorval cooling centrifuge) at 400g at 4°C for 30 min. The interface layer containing monocytes and lymphocytes was carefully aspirated and then placed in sterile tubes containing 2ml RPMI-1640 media (Gibco, Paisley, Scotland). Cells were washed 3 times in RPMI-1640 media centrifuged at 1800 r. p. m. at 4°C and then suspended in one ml of RPMI-1640 containing 10% fetal calf serum (FCS, Sigma, USA). Lymphocytes (1×10^6 /well) were added in 96 well tissue culture plates (Sarstedt, USA). Each well received 50 μ l PHA (Sigma-Aldrich, USA) (5 μ g/ml) as mitogen. The plate was incubated at 37°C, 5% CO₂ for 72h, in the humid incubator (Nuair, USA), then MTT solution was added in a ratio of 1/10 concentration of total sample then incubated at 37 °C, 5% CO₂ for 4h. Lysis buffer was then added (50 μ l/well) and plate incubated over night at 37°C, 5% CO₂. Absorbance values were recorded at 470nm using a microplate reader (Molecular Devices Corporation, USA). Stimulation index (SI) = the differences in reading between mean absorbance values of triplicate test cultures and mean absorbance values of corresponding triplicate of control rat.

Alkaline Comet Assay

The alkaline comet assay was applied to determine the percentage of damaged DNA concentration in the comet tail by measuring the total intensity of ethidium bromide fluorescence in the peripheral blood lymphocytes (Jaloszynski & Szyfter, 1999). For each cell, the length of the DNA migration (comet tail length) was measured in micrometers from the center of nucleus to the end of the tail. The percentage of the damaged DNA concentration in the comet tail was determined by measuring the total intensity of ethidium bromide fluorescence in the cells, which was taken as 100% and determining what percentage of this total intensity correspond to the intensity measured only in the tail.

Statistical Analysis

Data were presented as mean \pm SE. Data was statistically analyzed using Student's t-test or one-way analysis of variance (ANOVA). A *P* value of less than 0.05 was considered as statistically significant.

Results

Effect of clonazepam on level of IFN- γ and IL-10

The levels of the Th1 related cytokine (IFN- γ) were significantly reduced ($P \geq 0.01$; $P \geq 0.001$) in treated rats, grp2 and grp 3, respectively, during the period of the study that is at day 7, 15, 21 and during the 7 days withdrawal period as compared with the control group (Figure 1).

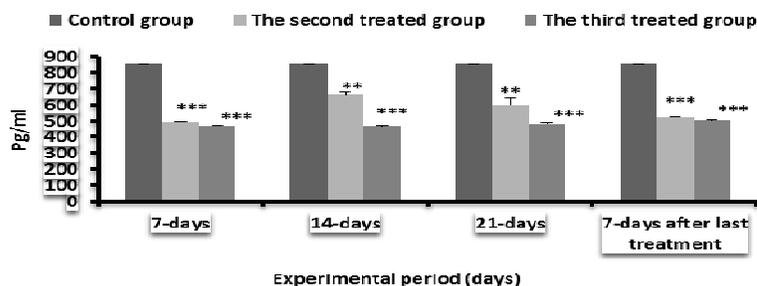


Figure 1. Level of IFN- γ in Serum of rats at various time intervals (days) of sub-chronic treatment and withdrawal period of clonazepam. The data are expressed as mean \pm S.E. ** $P \leq 0.01$, *** $P \leq 0.001$.

On other hand, the level of serum IL-10, Th2 related cytokine was not altered at day 7 following sub-dose of clonazepam (Figure 2). Notably, IL-10 levels were observed to be significantly elevated in both treated groups

($P \geq 0.01$; $P \geq 0.001$) as compared to control group at day 14 and 21 following dosage intake. However, during withdrawal period, IL-10 production decreased similar to the levels observed at day 7 (Figure 2).

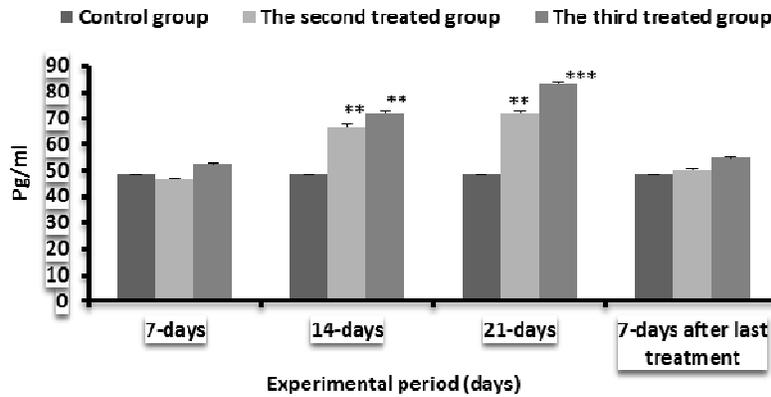


Figure 2. Level of IL-10 in Serum of rats after sub-chronic treatment and withdrawal period of clonazepam. The data are expressed as mean \pm S.E. ** $P \leq 0.01$, *** $P \leq 0.001$.

Effect of clonazepam intake on cortisol production

Serum cortisol levels were significantly decreased ($P \geq 0.05$; $P \geq 0.01$) in treated rats, grp2 and 3 than in controls during the

clonazepam treatment and its withdrawal period (Figure 3). However, the decrease in the level of cortisol was not correlated with increasing doses of clonazepam when compared to the controls.

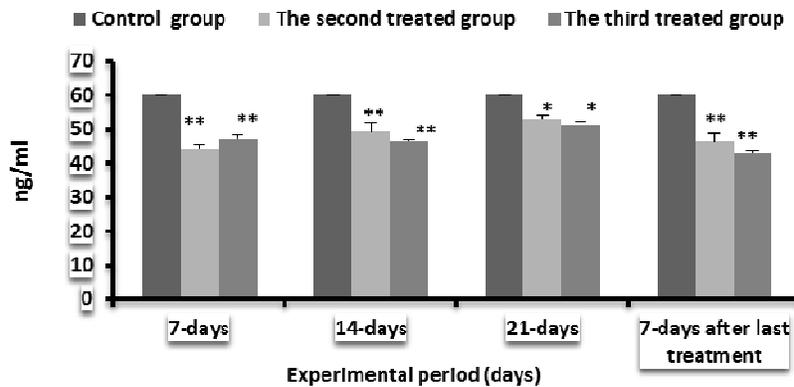


Figure 3. Level of cortisol in serum of rats after sub-chronic treatment and withdrawal period of clonazepam. The data are expressed as mean \pm S.E. ** $P \leq 0.05$, *** $P \leq 0.01$.

Effect of clonazepam on spleen cellularity

The total number of splenocytes after clonazepam administration were significantly ($P \geq 0.05$; $P \geq 0.01$) reduced throughout the

experimental period. Notably, spleen cellularity was decreased especially in the grp 3 than the grp 2 (Figure 4).

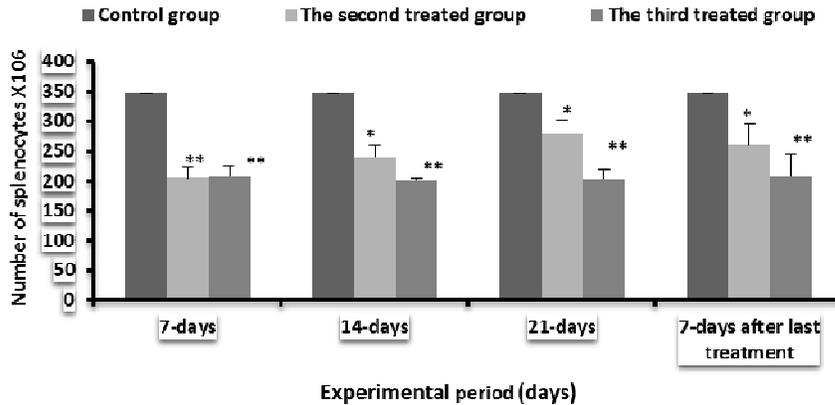


Figure 4. Total cell number of splenocytes of rats at various time intervals (days) after sub-chronic treatment and withdrawal period of clonazepam. The data are expressed as mean \pm S.E. ** $P \leq 0.05$, *** $P \leq 0.01$.

Effect of clonazepam on peripheral blood lymphocytes transformation

The results of PHA stimulated peripheral blood lymphocytes of treated rat illustrated a significant suppression in the lymphocytes transformation index ($P \geq 0.001$) in both treated groups (Figure 5). However, this suppression of lymphocytes was detected

especially in the grp3 more than the grp2 along the experimental period as compared to the control group. In addition, this suppression was in parallel and correlated with the increasing doses of clonazepam and maintained its effects during the withdrawal period.

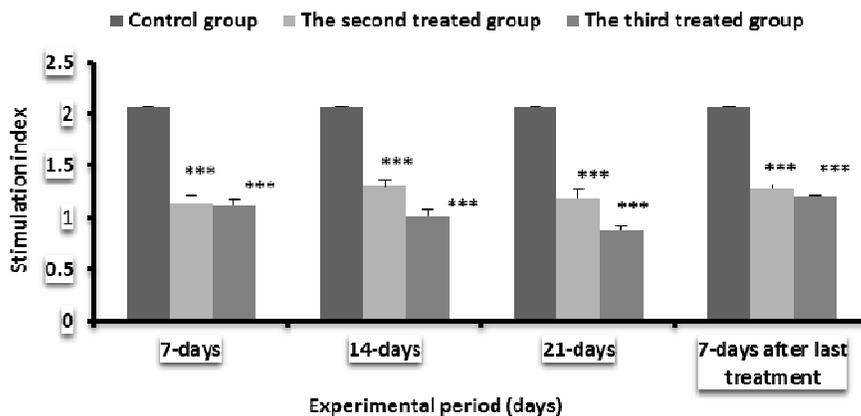


Figure 5. Proliferation response of peripheral blood lymphocytes of rats after concentration of PHA (5 μ g/ml) at various time intervals (days) of sub-chronic treatment and withdrawal period of clonazepam. The data are expressed as mean \pm S.E. *** $P \leq 0.001$.

Effect of clonazepam on the percentage of DNA degradation of peripheral blood lymphocytes

We observed a significant increase ($P \geq 0.001$) in comet tail length with increasing dosage of clonazepam administrated in both treated groups especially on day14 and 21 of the study (Figure 6) and (Pictures B-E). Meanwhile, the increase in comet tail length

were significantly reduced ($P \geq 0.05$; $P \geq 0.01$) on the 7th day and withdrawal period respectively, as compared to control group (Picture A). A significant elevation in comet tail DNA percentage ($P \geq 0.001$) was also observed after clonazepam treatment in both treated groups (Figures 7) and (Pictures F-I), but these elevation were significantly ($P \geq 0.01$) decreased during the withdrawal period versus the control group (Picture A).

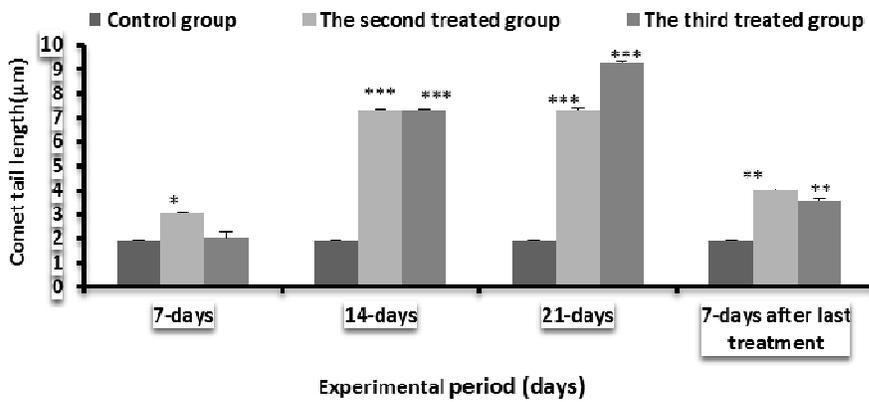


Figure 6. Effect of sub-chronic treatment and withdrawal period of clonazepam on the comet tail length (µm) of peripheral blood lymphocytes of rats. The data are expressed as mean ± S.E. * $P \leq 0.05$, ** $P \leq 0.01$ *** $P \leq 0.001$.

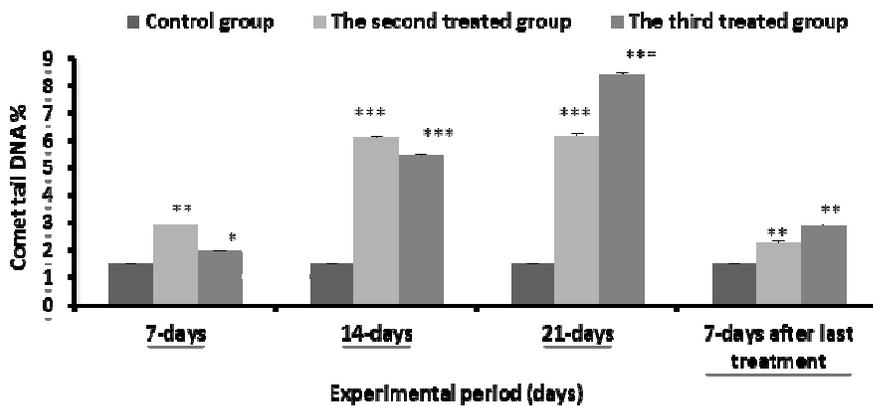
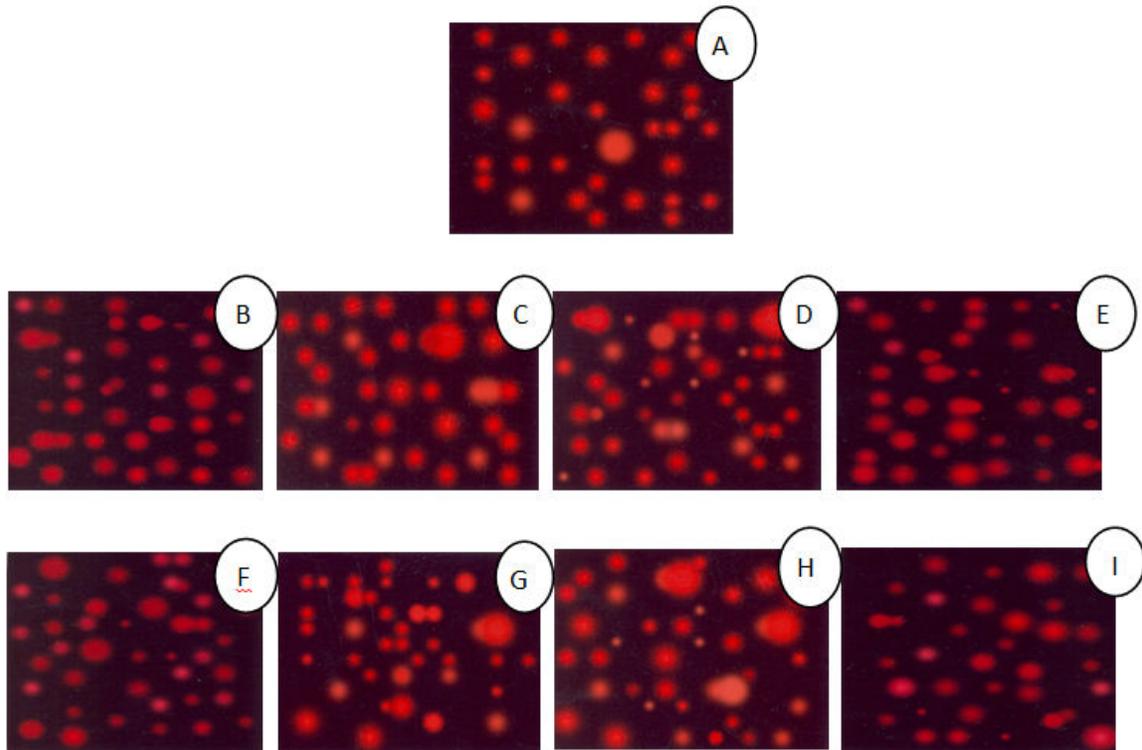


Figure 7. Effect of sub-chronic treatment and withdrawal period of clonazepam on the comet tail DNA (%) of peripheral blood lymphocytes of rats. The data are expressed as mean ± S.E. * $P \leq 0.05$, ** $P \leq 0.01$ *** $P \leq 0.001$.



Picture A: Peripheral blood lymphocytes of male albino rats in the control group.

Pictures B – E: The effects of sub-chronic doses of clonazepam administration and withdrawal period on peripheral blood lymphocytes of the second group (1st, 2nd, 3th weeks and withdrawal period, respectively).

Pictures F-I: The effects of sub-chronic doses of clonazepam administration and withdrawal period on peripheral blood lymphocytes of the third group (1st, 2nd, 3th weeks and withdrawal period, respectively).

Discussion

Benzodiazepines (BZPs) are widely used drugs as tranquilizers, anticonvulsants and in various other indications as light anesthesia and skeletal muscle relaxation. However, not all benzodiazepines have been tested on immune function (Huemer *et al.*, 2010).

Many BZPs induced prolonged impairment of cellular immune functions in experimental animals after chronic low dosage administration (Massoco & Palermo-Neto, 2003; Huemer *et al.*, 2010). In this study, we identified a significant suppression in the levels of IFN- γ and on the other hand, an

increase of IL-10 production in rats subjected to sub-chronic doses of clonazepam. These results are in agreement with those of Kalashnikov *et al.* (2002) who found that exposure to low doses of BDZ resulted in long-lasting reduction of TNF-alpha, IL-1, IL-6, IL-2 and IFN- γ . IL-10 is produced as part of the homeostatic response to inflammation whose critical role is in limiting the duration and intensity of immune and inflammatory reactions (Moore *et al.*, 2001).

The present work also showed the suppressive effects of clonazepam on cytokines production, spleen cellularity and lymphocyte mitogenic response in a dose

dependent pattern and duration of the drug administration. In experimental mice models, alprazolam induced severe inhibitory effects on the proliferative responses of both B- and T-cells (Chang *et al.*, 1991). It also reduced the production of IL-2 by splenic T-cells and IL-1 and TNF by peritoneal macrophages. Moreover, long-lasting depression of lymphocytic proliferation was also described in offspring of rats exposed to either diazepam or clonazepam during pregnancy (Schlumpf *et al.*, 1991). In a case report of pancytopenia, the drug toxic effects on differential WBC counts was observed, following oral administration of 0.25 mg clonazepam twice a day for approximately two weeks (Bautista-Quach *et al.*, 2010).

Benzodiazepine receptor sites (PBR) or TSPO play an important role in the regulatory processes and metabolic functions of the tissue in which such receptors are present (Zavala *et al.*, 1990; Marino *et al.*, 2001).

In the current study, the level of serum cortisol concentration in both treated groups showed a significant suppression along the experimental period. The immune toxic effects might be related to cortisol production. West *et al.*, (2001) stated that stimulation of PBR in steroidogenic tissues such as the adrenals increased glucocorticoid production. Glucocorticoid hormones are known for their potent immunosuppressive and anti-inflammatory properties. On the other hand, clonazepam was shown to counteract the effect of stress on cortisol level (Chevassus *et al.*, 2004).

Moreover, GABA and benzodiazepines reduce levels of HPA axis hormones, including CRF (corticotropin releasing hormone) and ACTH (adrenocorticotrophic hormone) and corticosterone (Arvat *et al.*, 2002) acting on CBR. Central pharmacological effects related to CBR acting by facilitating inhibitory GABA neurotransmission in the CNS, may regulate

the release of neuroendocrine hormones involved in the immune response to stress (Elmesallamy *et al.*, 2011).

In accordance with the present work, several reports identified PBR in endocrine steroidogenic tissues, organs and cells of the immune system, such as macrophages and lymphocytes. Thus, the PBR may be a possible primary target for the immunotoxic effects of BDZ (Righi *et al.*, 1999). In this context, the cytokine system emerges as a good candidate. Indeed, the production and release of cytokines are known to mediate both inflammatory and immune responses (Wiegers & Reul, 1998) and not only cortisol (Almawi *et al.*, 1996). Schlumpf *et al.*, (1994) reported that PBR stimulation of macrophage and lymphocyte membranes changed the cytokine network.

In the present study, the results record significant increase DNA damage in the peripheral blood lymphocytes of both treated groups throughout the experimental period. Our results are in coincident with some following investigators. An *in vitro* study carried out by Saha *et al.* (2009) concluded that alprazolam strongly interacted with DNA, resulting in conformational changes in the DNA. Moreover, Clonazepam was found to have a strong binding capacity to PBR in rat aortic smooth muscles compared to other benzodiazepines. These binding sites were concentrated in the mitochondria (Cox *et al.*, 1991). PBR bind with high affinity to cholesterol and transport it across the mitochondrial membrane (Papadopoulos *et al.*, 1997) and this may explain the appearance of foam cells and degeneration of smooth muscle observed in the aorta of clonazepam treated rats.

Interestingly, the action of the PBR ligands seems to be connected with blockage of voltage-dependent Ca²⁺ channels (Ostuni *et al.*, 2004). Calcium release appears to be essential for T cell activation, cytokine

synthesis, and proliferation; such increases and decreases in intracellular Ca^{2+} have been linked to apoptosis (Mason, 1999).

In addition, PBRs are involved in development of apoptosis (Ikonomidou *et al.*, 2000). The mitochondrial peripheral benzodiazepine receptor (mPBR) is involved in a functional structure designated as the permeability transition pore, which controls apoptosis. Binding of Fas/APO-1/CD95 triggers a prototypic apoptosis inducing pathway (Didier *et al.*, 2002) or through transmembrane mitochondrial potential ($\Delta\Psi_m$) dissipation (Chelli *et al.*, 2004).

Clonazepam can induce apoptosis through many mechanisms. One of these mechanisms is the peripheral non GABAA receptors. Another supposed mechanism is the oxidative stress induced by the overdose usually reached in the abuse and tolerance state, and alteration in the cell media via GABA receptors modulations of the ion channel influx and outflux activity causing change in normal chloride and other ion balance in the neuronal cells, and finally activation of the caspase system and eventually cell death apoptosis (Girgis *et al.*, 2010).

In conclusion, sub-chronic administration of clonazepam may adversely affect immune responses by altering-cytokine production and by suppressing lymphocyte function. Further, the results of the present study raise concern regarding the safety of benzodiazepine administration over prolonged duration of time.

Acknowledgements

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