CELLULAR AND DNA CHANGES DUE TO CLONAZEPAM ABUSE IN BRAINS OF ALBINO RATS AND ROLE OF CLONIDINE DURING WITHDRAWAL PERIOD

BY

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ABSTRACT

Clonazepam as an addictive drug was studied to elucidate its destructive effects on rats' brain. Also, the possible effect of clonidine was evaluated during withdrawal period. Sixty male albino rats were divided into three main groups. Group I (negative control group) comprised ten rats. Group II comprised forty rats which were divided into equal subgroups: group II a (Clonazepam dependent group), group II b (Clonazepam withdrawal group), group II c rats received clonidine during the withdrawal period for two weeks and group II d rats received clonidine during the withdrawal period for four weeks. Group III (Clonidine positive control group) comprised ten rats. Rats of all groups were sacrificed at the end of the designed period. Clonazepam dependent rats (group II a) showed the highest mean optical density values of brain DNA degradation compared to control. Group II c showed more improvement in DNA degradation than in dependence and withdrawal groups. Group II d showed much more improvement compared to groups II a, b, and c. Histopathology and histochemical results paralleled those found in DNA study. In conclusion, clonazepam should be prescribed cautiously as patients may turn addict to it. Also, clonidine should be more investigated as an adjuvant in clonazepam addiction withdrawal in human being.

INTRODUCTION

Recently, drugs are so commonly used and abused that virtually everyone has some familiarity with the concepts of drug addiction, misuse and abuse (Taylor, 2008). Drug abuse is now one of the major health problems. It is implicated in many deaths, both directly from overdose and indirectly as a result of injuries sustained while the individual is intoxicated or due to serious transmitted infections (Laine et al., 2001).

Benzodiazepines are among the most commonly prescribed drugs worldwide; whose concurrent abuse is a major clinical problem, especially among street addicts (O'Brien, 2005).

They are widely prescribed for a variety of conditions, particularly anxiety and insomnia. Whenever used chronically, benzodiazepines can cause addiction (Lance and Brian, 2000). While relatively few patients who receive benzodiazepines for medical indications may abuse their medication, there are individuals who specifically seek benzodiazepines for their ability to produce a "high" (Marriott and Tyrer, 2001).

Clonazepam is a synthetic benzodiazepine derivative used to treat panic disorder and certain types of seizures. It has the potential to be abused and may cause addiction or dependence after long-term use or in high doses. Abuse is more likely to occur in people with a history of alcohol or drug addiction (Heberlein et al., 2009). It 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, 2004).

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).

Clonidine has been used to mitigate or prevent as well as to treat signs of withdrawal from tobacco, alcohol (Kosten and O'Connor, 2003), narcotics (Umbricht et al., 2003) and opioids (Chen et al., 2007). It may help to ameliorate some of the adverse sympathetic nervous activity associated with withdrawal from these agents, as well as decrease craving for the drug by decreasing sympathetic outflow in the central nervous system, thereby diminishing tachycardia, sweating, and tremors (Donald et al., 2002).

Opioids, benzodiazepines, barbiturates and anticholinergics were considered the most commonly abused drugs in Menoufiya governorate where most of dependents used codeine, clonazepam, phenobarbital and trihexyphenidyl (Ahmed, 2003).

Among methods used for the evaluation of cytotoxicity of chemicals, measurement of apoptosis has become an essential component, through knowing toxic mechanism (Sperandio et al., 2000).

The aim of the present study is to elucidate the destructive effect of clonazepam dependence on rats' brain and detection of possible role of clonidine in alleviating the withdrawal manifestations and brain damage depending on manifestations, histopathological, histochemical and DNA degradation by gel electrophoresis methods.

MATERIAL AND METHODS

Animals:

The study was carried out on sixty adult male albino rats of Sprague species 150-200 gram average weight obtained from the animal house in Menoufiya governorate. They were left to acclimatize for one week. They were fed on ordinary food and housed under standard laboratory conditions.

Drugs :

- Clonazepam (Apetryl tablets 2 mg) obtained from multi Apex pharma S.A.E. Bader city, Cairo, Egypt.
- Clonidine hydrochloride: (Catapres tablets 150 μg), obtained from Boehringer Ingleheim Co.

Groups:

The animals were divided into 3 main groups as follows:

Group I (Negative control):

Consisted of ten rats kept without any drugs, throughout the experiment.

Group II (Clonazepam treated group): Experimental group of forty rats were given clonazepam. Dependence to clonazepam was induced starting with therapeutic doses for rats according to Paget and Barnes (1964), considering that therapeutic dose for human is 4mg/day (Martindale, 2008). So, the calculated starting dose for rat was 0.36 mg/kg. The clonazepam dose was gradually increased by adding the initial calculated therapeutic dose every three days till the end of the month.

The calculated clonazepam dose was mixed with a small amount of food (a piece of bread) and given orally to each animal.

After the end of the month, clonazepam dependent rats were divided into 4 equal subgroups:

Group II a (Clonazepam dependent group): ten rats were sacrificed at the end of the month.

Group II b (Clonazepam withdrawal group): Ten rats were left for an extra one month without taking clonazepam then sacrificed.

Group II c (Clonidine two weeks treated group): Ten rats were given clonidine for two weeks in the withdrawal period of clonazepam, according to a clonidine regimen, and then sacrificed.

Group II d (Clonidine four weeks treated group): Ten rats were given clonidine for four weeks in the withdrawal period of clonazepam, according to a clonidine regimen, and then sacrificed.

Clonidine regimen: rats were given half the therapeutic dose of clonidine hydrochloride 13.5 μg/kg rat BW for two days

after stoppage of clonazepam in withdrawal period by oral route. Then the full therapeutic dose (27 μg/kg rat BW) was given for the following days, then after half the therapeutic dose was given for the last 2 days of both group II c & group II d. This regimen was designed to avoid hypotension and syncope if the full therapeutic dose was started upon first (El-Seidy, 2005). The dose was calculated on the same principles according to Paget and Barnes (1964), considering the human therapeutic dose of clonidine to be 300 μg /day (Schonwald, 2001).

Group III (Clonidine positive control group):

Ten rats were given clonidine in the same schedule for thirty days, and were sacrificed at the end of the month.

After elapse of the prescribed time of drug administration to each group, the animals were sacrificed by cervical dislocation and the brain was extracted and divided equally and symmetrically into its two halves. One half was used for histopathological and histochemical studies, the other was put in Eppendorf tube and frozen at-20°C for DNA study.

Histological methods:

1- Hematoxylin and Eosin (Hx & E) stain was used for histological examination (Drury and Wallington, 1980).

2- Masson's trichrome (MT) stain was used to demonstrate the collagen fibers "stained blue" (Kiernan, 1999).

3-Toludine blue (TB) method to demonstrate Nissl's substance (Bancroft et al., 1990).

Histochemical study: (Bancroft et al., 1990).

A- Periodic Acid-Schiff (PAS) for neutral mucosubstances.

B- Methyl green pyronin (MGP) for Deoxyribonucleic acid (DNA) and Ribonucleic acid (RNA).

DNA study:

(1) Gel preparation

It was prepared with 1.8% electrophoretic grade agarose (BRL). The agarose was boiled with tris borate EDTA buffer (1 x TBE buffer. 89 mM Tris, mM boric acid, 2mM EDTA, pH 8.3). Ethidium bromide (0.5 microgram\ml) was added to the gel at 40°C. Gel was poured and allowed to solidify at room temperature for one hour before loading of samples.

(2) DNA damage detection in tissues:

(A) DNA extraction:

According to Aljanabi and Martinez, (1997) with Hassab El-Nabi modification (2004). To detect DNA damage, the gel was visualized using a 312 nm UV light under a transilluminator, photographed using a Polaroid camera.

(B) Apoptosis analysis:

Apoptotic bands were located at 200 base pair (BP) and its multiples. The intensity of apoptotic bands could be measured by Gel-Pro program so the fine apoptotic bands could be detected even in controls, as maximum optical density values.

STATISTICAL ANALYSIS

The data were analyzed using IBM computer and SPSS 17.0 for Windows statistical package. Mean, standard deviations, and t-test were used. The level of significance was set as P values <0.05, and high significance when P values were <0.001.

RESULTS

I- Observed behavioral changes and manifestations:

Group I (Negative control): normal behaviour.

Group II: Forty rats which were given gradually increasing doses exhibited prolonged sleeping periods at the end of 1st week, and then began to be lethargic and less active. Rats began to seek for the dose diet and showed aggressiveness before the diet time through the 3rd week, and finally they looked ill, with loss of appetite and body weight. The manifestations were the same for group IIa.

Group II b (Clonazepam withdrawal group): Rats became irritable and restless from the 2nd and 3rd day after clonazepam has been stopped. Rats became aggressive, hostile to one another, screaming most of the time with loss of appetite and body weight. They exhibited chills, and some of them had convulsions that increased in frequency by time. Manifestations were improving during the 2nd and 3rd week and they became almost nearly normal by the end of 4th week.

Group II c and group II d (clonidine treated group for 2 and 4 weeks respectively): Rats showed less manifestations. The rats began to thrive early in the first week.

Group III (Clonidine positive control group): no abnormal manifestations were observed.

II- Histopathological results :

Control cerebral cortex of rats was formed of six indistinct laminae differentiated from each other by the type, density, and arrangement of cells. (Fig. I, sec. 1)

Histopathological findings include vascular congestion of meningeal, cerebral and cerebellar vessels together with disruption of normal arrangement of cell layers in both cerebrum and cerebellum in clonazepam dependent rats. The deeply eosinophilic staining of neuronal cell body, nuclear fragments and cytoplasmic buds which contain the nuclear fragments

in both cerebral and cerebellar cortex are criteria of neuronal apoptosis (Fig. I, sec.3).

Meanwhile, the clonazepam withdrawal group showed moderate dilatation and congestion of the cerebral vessels with patchy disruption of normal arrangement of cell layers. Numerous neurons were shrunken and surrounded by vacuoles containing few Nissl's granules while some others appeared normal (Fig. I, sec. 5).

Brains of rats treated with clonidine for two weeks during the withdrawal period of clonazepam showed few neuroglial cells and shrunken neurons surrounded by vacuoles together with numerous normal neurons containing Nissl's granules. They also showed degeneration of few Purkinje cells, while other Purkinje cells appeared normal (Fig. II, sec. 1 MT stain & sec. 2 TB stain).

Regarding rats received clonidine for four weeks during withdrawal period, their brains showed a picture more or less similar to control (Fig. II, sec. 4 MT stain).

III-Histochemical findings

A) PAS reaction:

The dependent group showed strong PAS reaction in red blood corpuscles (RBCs) inside dilated congested blood vessels together with mild reaction in degenerated neurons. There was moderate increase in the PAS reaction in other neurons and Purkinje cells (Fig. III, sec. 2, 3).

Meanwhile, clonazepam withdrawal group showed intense PAS reaction in RBCs inside dilated congested blood vessels and disrupted neurons in rat's brains (Fig. III, sec. 4).

The group that received clonidine for two weeks during the withdrawal period of clonazepam showed slightly better picture than those rats after stopping clonazepam administration abruptly. Rat's brains showed moderate reaction in shrunken neurons surrounded by vacuoles. The cerebellum showed strong reaction of few Purkinje cells, while other Purkinje cells and granular layer appeared normal (Fig. III, sec. 5). With continuing clonidine treatment for four weeks during the withdrawal period of clonazepam, rats showed a picture similar to control (Fig. III, sec. 6).

B) MGP stain (DNA histochemistry):

Clonazepam dependent rats showed patchy decrease in the amount of nucleic acids more pronounced in vacuolated and necrotic neurons and degenerated Purkinje cells (Fig. IV, sec. 2).

Rats' brains of the withdrawal group stained with MGP (Fig. IV, sec. 3) showed patches of decreased nucleic acids in vacuolated and necrotic neurons and degener-

ated Purkinje cells. These similar criteria to the pervious group were less in extent and the patches were in aggregates rather than distributions.

Rats treated with clonidine for two weeks during the withdrawal period of clonazepam were slightly better than those rats after stopping clonazepam administration abruptly. There was moderate DNA and RNA content in both cerebrum and cerebellum (Fig. IV, sec. 4). With continuing clonidine treatment for four weeks during the withdrawal period of clonazepam, rats brains showed a picture similar to controls (Fig. IV, sec. 5).

IV- DNA study:

Figure (V) showed control lane and different 4 lanes that represent different groups. Lanes obviously illustrate the pattern and degrees of DNA degradation via ladders.

Computer Gel pro- analyzer :

The chart represented the pattern of DNA degradation photographed in the lanes (Fig. V), and was divided into three areas:

1-Area of intact DNA which comprises lengths between 1000 to 0 base pairs (BP).

2-Area of DNA degradations (Apoptosis) which contains fragments between 1000- 200 BP.

3-Area for RNA (100 BP).

DNA degradation area:

It showed the changes indicative of damage to the nuclear DNA of the affected brains. The area between 1000-0 BP was quite normal in the control group charts (Bichart of lane 1 & fig. V).

Rats that turned dependent for clonazepam (Group II a) had brain DNA degradation to the highest extent in areas of the chart corresponding to 600-400-200 BP (Bichart of lane 2 & fig. V).

Brain specimens of the rats after the withdrawal (Group II b) showed DNA damage which is somewhat similar to that in group II a but to a little extent (Bichart of lane 3 & fig. V). Rats that received clonidine for two weeks after being dependent (Group II c) showed improved damage of DNA than in the withdrawal group and dependence group as well (Bichart of lane 4 & fig. V). Rats that received clonidine for four weeks after dependence (Group II d) showed very little DNA degradation and were not so different than in the normal group (Bichart of lane 5& fig. V).

Statistical analysis:

Table (1) showed that the mean optical densities of the DNA degradation in Group II a at 200, 400, and 600 BP were significantly higher than the corresponding BP of the control group ($p < 0.001$).

Table (2) revealed that the mean optical

densities of DNA degradation in the withdrawal group were significantly higher than that of the control group (P<0.001).

Table (3) showed that the DNA degradation in Group II d (received clonidine for four weeks after withdrawal) was significantly less than in Group II c (received clonidine for two weeks).

Table (4) showed insignificant difference in the DNA degradation pattern at 200, 400, 600 BP in clonidine treated Group III compared to that of control Group I (P >0.05).

DISCUSSION

As many as addictive substances are abused, there are a number of drugs that are used, misused and abused as well (Koob and Le-Moal, 2001). Among drugs that are prescribed for medicinal purposes then patients turn to abusers, are barbiturates, benzodiazepines, cough sedatives, narcotic analgesics... etc. While these drugs are abused by patients, they are also known and spreaded by dealers and drug handlers for youths to produce a high (Lance and Brian, 2000). 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., 2000).

Benzodiazepines bind to benzodiazepine receptors on $GABA_A$ complexes to increase the affinity of GABA for its receptor and to increase the frequency of Cl- channel opening in response to GABA binding. Benzodiazepines also inhibit adenosine uptake apart from $GABA_A$ activity (Sieghart, 1995).

Clonazepam, a recently developed benzodiazepine derivative is nowadays widely prescribed for a variety of psychiatric and neurologic conditions. 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 (Juergens, 2004).

In the present study, clonazepam is studied to elucidate its destructive effects on rats' brain by histopathology, histochemistry and DNA degradation apoptosis by Gel Electrophoresis. Clonazepam (given in the designed gradually increasing doses), achieved the aim of inducing tolerance and dependence to the experimental animals. Addiction versus withdrawal syndrome were carefully studied on the bases of clinical and behavioral manifestations, together with histopathology, histochemistry and DNA changes. Also, the role of clonidine (an alpha receptor agonist previously used for hypertension management) was evaluated to detect its possible effect on improving the withdrawal symptoms and signs. Nevertheless, it was used with special regimen to

avoid syncope and hypotension if full therapeutic dose had been used from the start.

The observed manifestations of clonazepm addiction in the present study together with withdrawal manifestations were consistent with findings conducted by Rudolf et al. (2002).

Histopathological examination of rats' cereberal cortex of group II a revealed disruption of normal arrangement of cell layers indicating the destructive outcome. The deeply eosinophilic staining of neuronal cell body, nuclear fragments and cytoplasmic buds which contain the nuclear fragments in both cerebral and cerebellar cortex may indicate degenerating or dying neurons (apoptotic cells) as has been previously reported by Abdel-Rahman et al. (2002).

These findings were similar to those found in the study conducted by Bittigau et al. (2002) who showed that phenobarbital, diazepam and clonazepam caused widespread apoptotic neurodegeneration in the brains of rats by electron microscope. Also, the detected gliosis might be a sign of neuronal abnormalities as had been previously reported by Rahmy and Hassona (2004). Another explanation for increased glial cells could be attributed to the role of these cells in degenerative processes of CNS (Reali et al., 2005).

Histopathological findings in clonazepam withdrawal group (group IIb) were similar to a little extent to the dependence group.

Rats' brain in the group treated with clonidine for two weeks during the withdrawal period of clonazepam showed degeneration of few Purkinje cells, while other Purkinje cells appeared normal. These findings reflect the improvement that accompanied clonidine in withdrawal period. The histopathological changes were improved more in rats' brains in the group received clonidine for four weeks with a picture more or less similar to controls. Bittigau et al. (2002) found similar inhibitory effect of apoptosis induced by diazepam (a derivative of benzodiazepines), by further administration of the benzodiazepine receptor antagonist flumazenil.

Regarding histochemical changes, the alteration observed in dependent group, denoted an increase in amount of mucopolysaccharides (MPS). This increase in MPS could be explained by inability of affected neurons to utilize existing glycogen due to impaired cell function, a manifestation of neuronal abnormality (Rahmy and Hassona, 2004).

After withdrawal of clonazepam, intense PAS were coincident with the patho-

logical picture of the same group. Rats received clonidine for two weeks during the withdrawal period of clonazepam were slightly better than those rats after stopping clonazepam administration abruptly. Rat's brains showed moderate reaction in shrunken neurons surrounded by vacuoles. With continuing clonidine treatment for four weeks during the withdrawal period of clonazepam, rats showed a picture similar to control group.

Clonazepam dependent rats showed patchy decrease in the amount of nucleic acids, more pronounced in vacuolated and necrotic neurons and degenerated Purkinje cells by MGP stain. The vitality of cells depends on the amount of nucleic acids present. DNA is the active nuclear material whose vital cytoplasmic action is protein synthesis mediated through RNA (Karp et al., 1991). This decrease coincided with degenerative changes histologically detected. It reflects the lost vitality of the neurons and inhibition of protein synthesis. This can be due to DNA fragmentation and chromatin condensation during apoptosis (Zou et al., 1998).

Clonazepam withdrawal group showed a more or less aggregates rather than diffuse pattern of reduced nucleic acid in rats' brain. Meanwhile, the picture was improved in group IIc, and IId as the clonidine was given for two and four weeks respectively.

Measurement of apoptosis has become an essential component of the evaluation of cytotoxicity of chemicals (Sperandio et al., 2000).

Gradually increasing doses of clonazepam with starting dose of 0.36 mg/kg were used to induce dependence in rats of the present study. Bittigau et al. (2002) had found that the threshold dose for triggering apoptotic brain damage was 0.5 mg/kg for clonazepam.

DNA study by Gel Electrophoresis showed fragmentation which is observed at 200, 400, and 600 BP. Visual evaluation of the different Lanes for control, dependence group, withdrawal, clonidine treatment for 2 and 4 weeks was performed, regarding number, width, intensity and distance of fragmentation bands from positive pole. These observations were confirmed by visual analysis of DNA ladder of degaradation. Also, Gel Proanalyzer program Bicharts and data were statistically analysed. It was noticed that clonazepam dependent group showed massive DNA degradation, compared to control group. Also, withdrawal group showed less degradation (apoptosis) than dependence group yet still higher than normal. In addition, clonidine treated group for two weeks showed less apoptosis than dependent group (group II a) although still more than control. There was more improvement in 4 weeks treated group

as it was nearly similar to control one.

The process of apoptosis can be explained by the depressant effect of the drug to an endogenous neuroprotective system in the brain that is crucial for neuronal survival (Hengartner, 2000; Huang and Reichardt, 2001).

In addition to the $GABA_A$ receptor-associated "central" benzodiazepine receptors, two other types of benzodiazepine binding sites are present in the brains which are called "peripheral" benzodiazepine binding sites, localized on the outer mitochondrial membrane of many tissues, including brain (Verma and Snyder, 1989) and the "micromolar" binding sites (Bowling and De-Lorenzo, 1993). Peripheral benzodiazepine receptors 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 psim $\delta\Psi m$) dissipation (Chelli et al., 2004).

Benzodiazepine group is one of the important oxidations ascribed to the cytochrome P family (Rose and Hodgson, 2004), which can trigger the apoptotic phenomenon (Mattson, 2000). Moreover, benzodiazepines affects the release of the vital neurotransmitters essential for the maintenance of the vitality of the nerve cells as well as other tissue cells. This affection alters the cells medium and pushes mechanisms of internal apoptosis. Caspases and mitochondrial cytochrome C release can be the underlying mechanisms (Vaux, 2002).

Clonazepam can induce apoptosis through many mechanisms. One of these mechanisms is the peripheral non $GABA_A$ 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 (Uren et al., 2000).

In the present study, it was found that clonidine could improve the withdrawal manifestations of clonazepam. It was previously described for treating the withdrawal manifestations in alcohol, opioids, tobacco and narcotics. The presumed mechanism may be due to its alpha adrenergic agonist effects that can greatly diminish the withdrawal criteria (Nelson, 2006).

In the present study, clonidine had been found to diminish greatly the apoptosis during the withdrawal period. Ilinykh et al. (2008) had found that clonidine decreased the amount of proapoptotic protein Bax mRNA in hippocampus of three days old rat pups brains, together with increasing the content of antiapoptotic protein Bcl-XL mRNA. These two mechanisms were concluded to be the cause of neuroprotective features of clonidine.

In conclusion, the present study revealed the possible effect of clonidine in alleviating the withdrawal manifestations of clonazepam and also its capability of neuroprotection via decreasing the brain apoptosis observed during clonazepam withdrawal in rats' brain. So, more investigations should be carried out to test value of clonidine in clonazepam withdrawal period in human subjects.

Table (1): Comparison between Group I (control) & Group II a (clonazepam dependent) regarding intact DNA and mean optical densities of DNA degradation at different base pairs (BP).

Parameters	Group I (n=10) X±SD	Group II a (n=10) X±SD	T test	P value
Intact DNA	164.9±2.269	8.0±1.41	163.48	<0.001
Band 200 BP	2.34±0.29	160.04±2.19	225.37	<0.001
Band 400 BP	1.79±0.15	154.62±0.37	1208.58	<0.001
Band 600 BP	6.53±0.22	80.7±0.59	371.09	<0.001

P < 0.001 highly significant

Table (2): Comparison between Group I (control) & Group II b (clonazepam withdrawal) regarding intact DNA and mean optical densities of DNA degradation at different base pairs (BP).

Parameters	Group I (n=10) X±SD	Group II b X±SD	T test	P value
Intact DNA	164.9±2.69	18.4	147.07	<0.001
Band 200 BP	2.34±0.29	65.33±3.33	59.67	<0.001
Band 400 BP	1.79±0.15	50.26±0.27	499.56	<0.001
Band 600 BP	6.53±0.22	73.46±0.38	478.69	<0.001

P < 0.001 highly significant

Table (3): Comparison between Group II c (clonidine two weeks treated) & Group II d (clonidine four weeks treated) regarding intact DNA & DNA degradation pattern at different base pairs (BP).

Parameters	Group II c (n=10) X±SD	Group II d (n=10) X±SD	T test	P value
Intact DNA	116.1±1.19	139.0±2.36	27.39	<0.001
Band 200 BP	29.5±0.32	7.39±0.35	146.25	<0.001
Band 400 BP	21.34±0.46	12.33±0.48	42.93	<0.001
Band 600 BP	39.3±0.56	20.35±0.51	79.1	<0.001

$P < 0.001$ highly significant

Table (4): Comparison between Group I (negative control) & Group III (clonidine positive control) regarding intact DNA and DNA degradation pattern at different base pairs (BP).

Parameters	Group I (n=10) X±SD	GIII (clonidine) (n=10) X±SD	T test	P value
Intact DNA	164.9±2.269	166.5 ±1.43	1.66	>0.05
Band 200 BP	2.34±0.29	2.51 ±0.36	1.17	>0.05
Band 400 BP	1.79±0.15	1.79±0.13	0.02	>0.05
Band 600 BP	6.53±0.22	6.67 ±0.23	1.43	>0.05

$P > 0.05$ not significant

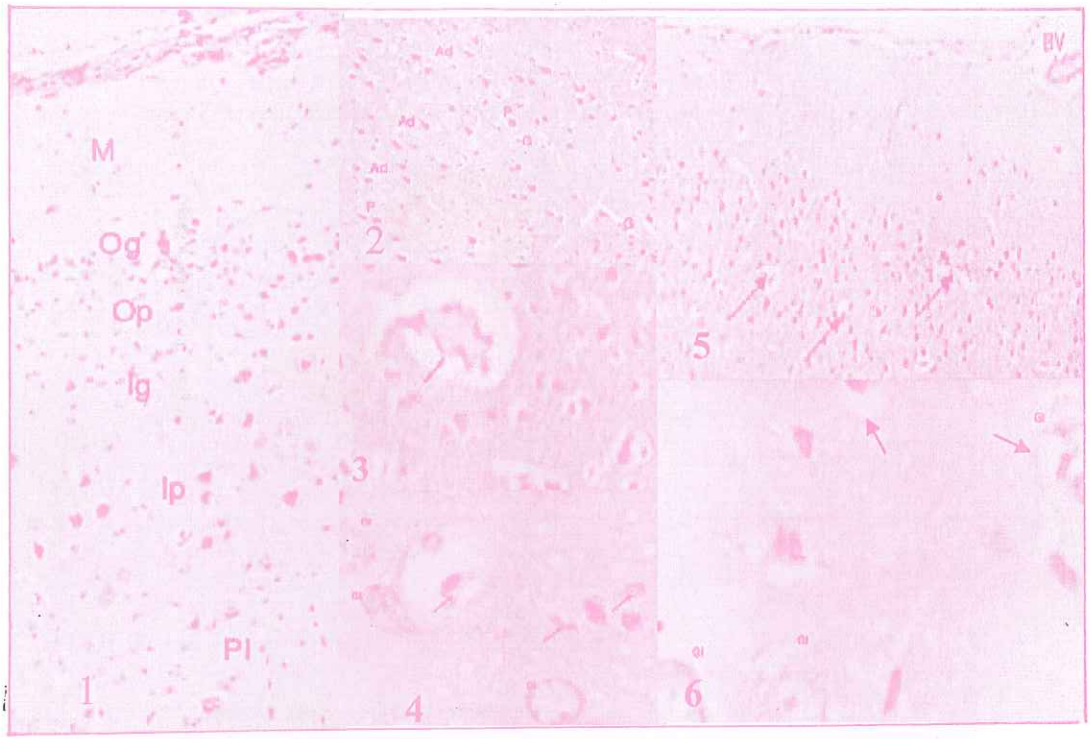


Fig. I :

(1) Section of control adult male albino rat cerebral cortex showing normal appearance of the all six layers; molecular (M), outer granular (Og), outer pyramidal (Op), inner granular (Ig), inner pyramidal (Ip), polymorphous layer (Pl). *(Hx & Ex 100)*

(2) Section of control adult male albino rat cerebral cortex showing normal appearance of scattered small, medium sized and large pyramidal cells (p); apical dendrite (Ad) and scattered granular cells (G). *(MT x 100)*

(3) Section of clonazepam treated rat cerebral cortex showing marked shrinkage of neurons surrounded by vacuoles and large macrophage is seen inside a large cavity (arrow). *(Hx& Ex 400)*

(4) Section of clonazepam treated rat cerebral cortex showing marked shrinkage of pyramidal cells (arrows) containing few Nissl's granules (lightly blue stained) and scattered glial cells (Gl). *(TB x 1000)*

(5) Section of rat cerebral cortex after stoppage of clonazepam treatment showing mild congested cerebral blood vessels (BV) and the presence of some shrunken neurons surrounded by vacuoles (arrows). *(Hx & E x 100)*

(6) Section of rat cerebral cortex after stoppage of clonazepam treatment showing mild shrinkage of pyramidal cells (arrows) containing Nissl's granules (moderately blue stained) and scattered glial cells (Gl). *(TB x 1000)*

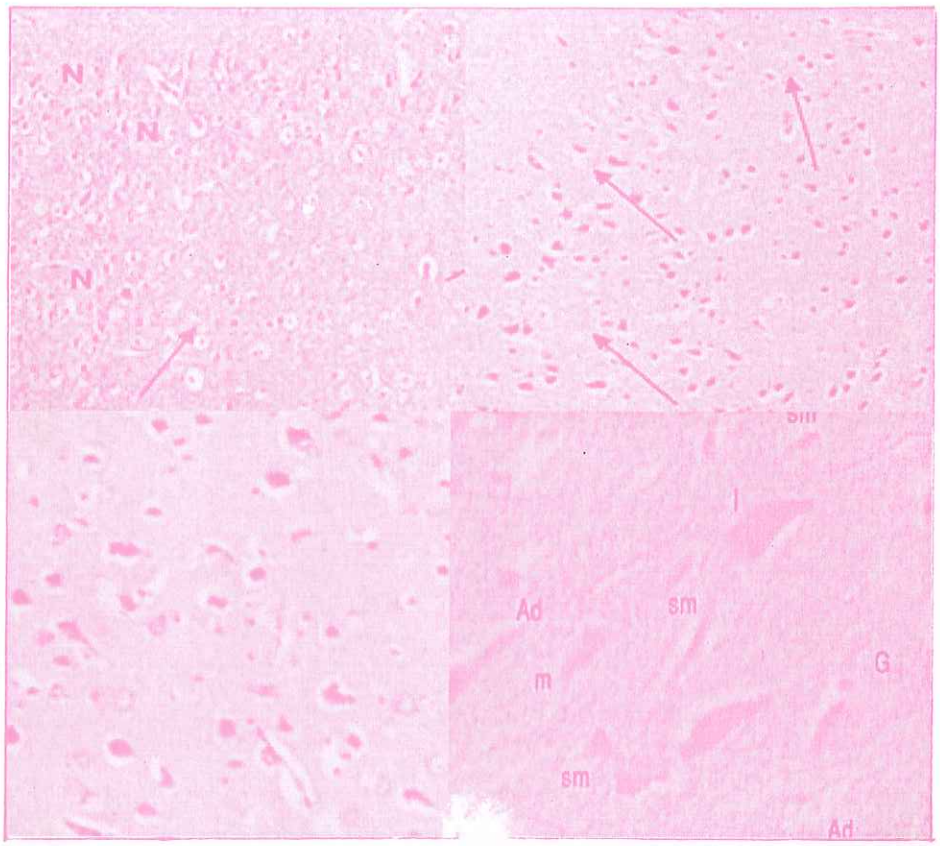


Fig. II :

(1) Section of rat cerebral cortex treated with clonidine 2 weeks after stoppage of clonazepam showing the presence of some normal neurons (N) & few shrunken neurons surrounded by vacuoles (arrow). *(MTx 100)*

(2) Section of rat cerebral cortex treated with clonidine 2 weeks after stoppage of clonazepam showing the presence of pyramidal cells containing Nissl's granules (moderately blue stained) & few shrunken neurons surrounded by vacuoles (arrows). *(TB x 100)*

(3) Section of rat cerebral cortex treated with clonidine (4 weeks) showing scattered various pyramidal cells of different sizes containing Nissl's granules (strongly blue stained). *(TB x 100)*

(4) Section of rat cerebral cortex treated with clonidine 4 weeks after stoppage of clonazepam showing normal appearance of scattered small (sm), medium sized (m) and large (l) pyramidal cells, showing apical dendrite (Ad) and scattered granular cells (G). *(MTx 400)*

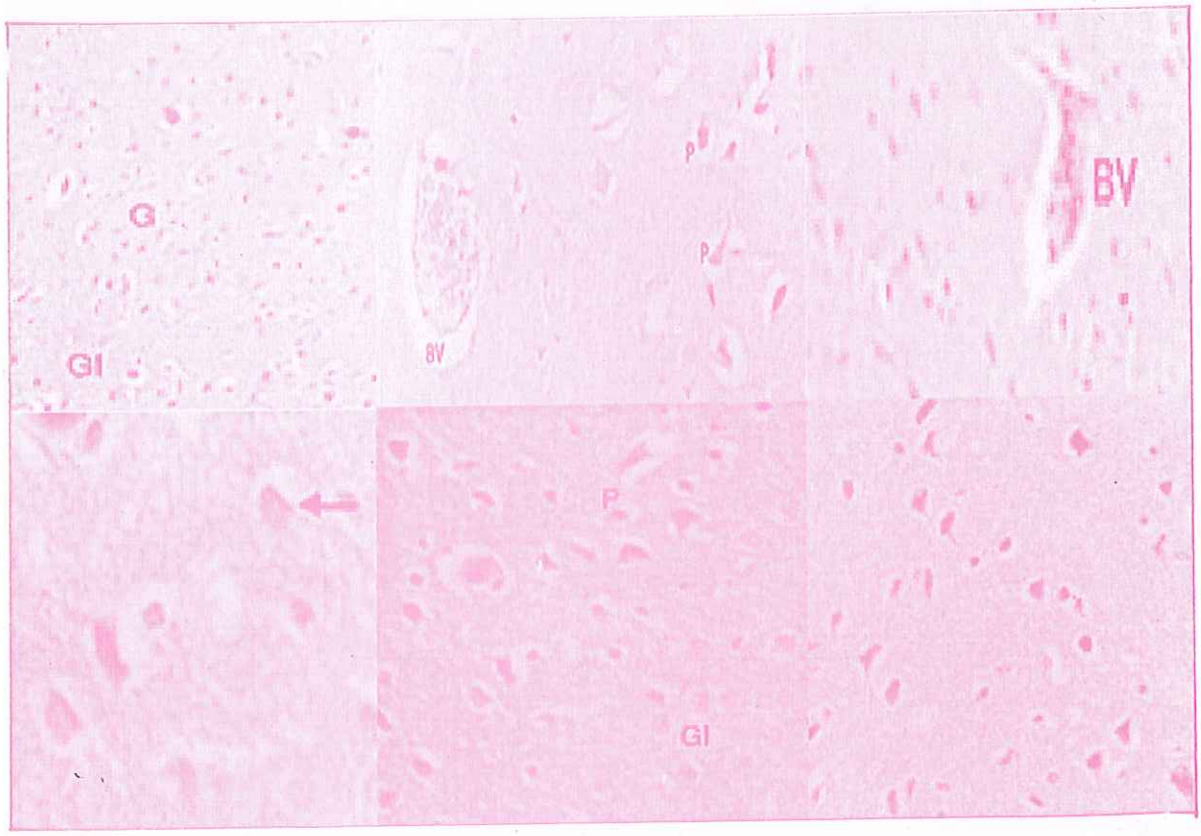


Fig. III :

(1) Section of control adult male albino rat cerebral cortex showing strong PAS reaction in pyramidal cells, moderate reaction in granular cells (G) and nerve fibers & weak reaction in glial cells (Gl). *(PAS x 100)*

(2&3) Section of clonazepam treated rat cerebral cortex showing congested cerebral blood vessels (BV) strongly stained together with strong reaction in scattered shrunken pyramidal cells (P). *(PAS x 100)*

(4) Section of rat cerebral cortex after stoppage of clonazepam treatment showing strong PAS reaction in scattered pyramidal cells and mild reaction in some degenerated ones (arrow). *(PAS x 400)*

(5) Section of rat cerebral cortex treated with clonidine 2 weeks after stoppage of clonazepam showing strong PAS reaction in pyramidal cells (P), moderate reaction in granular cells (G) and nerve fibers & weak reaction in glial cells (Gl). *(PAS x 200)*

(6) Section of rat cerebral cortex treated with clonidine 4 weeks after stoppage of clonazepam showing strong PAS reaction in scattered pyramidal cells. *(PAS x 200)*

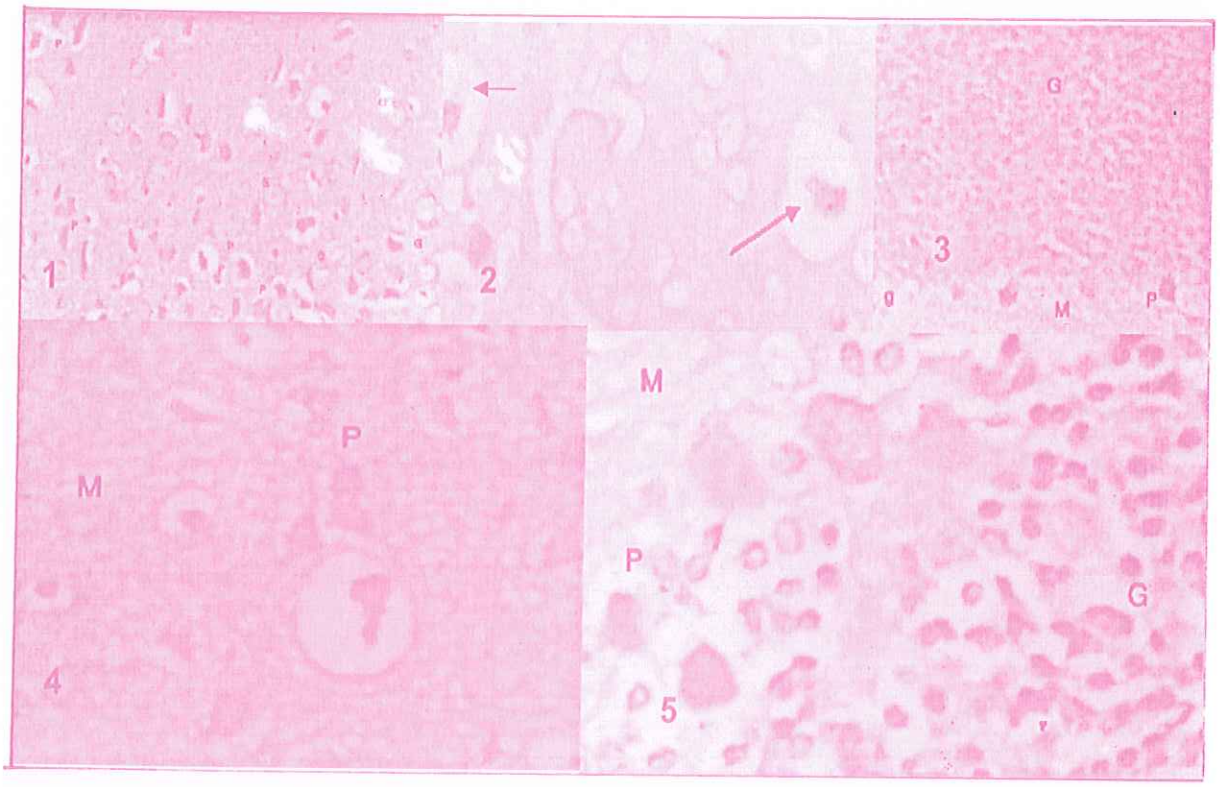


Fig. IV :

(1) Section of control adult male albino rat cerebral cortex showing moderate DNA and RNA reaction in pyramidal cells (p); moderate reaction in granular cells (G) and nerve fibres. *(MGP x 400)*

(2) Section of clonazepam treated rat cerebral cortex showing mild to moderate DNA and RNA reaction in degenerated shrunken neurons and moderate reaction in macrophages present in cavities (arrows). *(MGP x 400)*

(3) Section of rat brain after stoppage of clonazepam treatment showing strong DNA and RNA reaction in Purkinje cells (P) apparently arranged in one row, weak reaction in degenerated Purkinje cell or ghost cell (g); moderate reaction in granular cells (G) and mild reaction in molecular layer (M). *(MGP x 200)*

(4) Section of rat cerebral cortex treated with clonidine 2 weeks after stoppage of clonazepam showing moderate reaction in scattered pyramidal cells (P) and in nerve fibers of molecular layer (M). *(Mx 400)*

(5) Section of rat brain treated with clonidine 4 weeks after stoppage of clonazepam showing moderate reaction in Purkinje cells (P) apparently arranged in one row; moderate reaction in granular cells (G) and mild reaction in nerve fibers of molecular layer (M). *(MGP x 400)*

Fig. (V) : Pattern of DNA and degress of DNA degradation in different study groups.

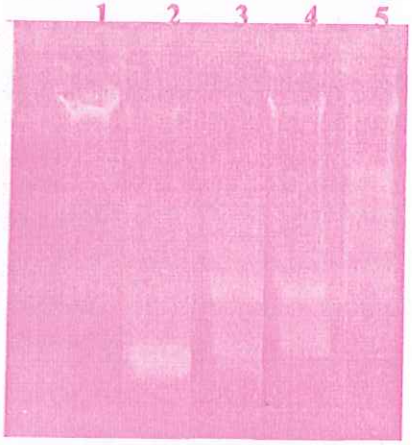
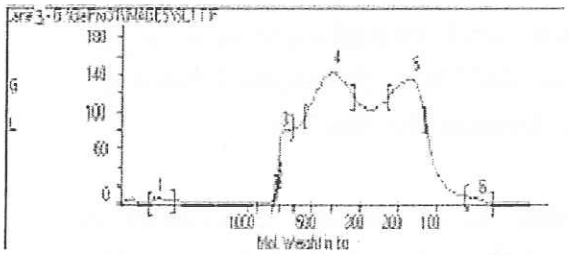
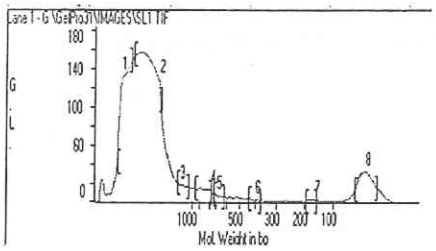


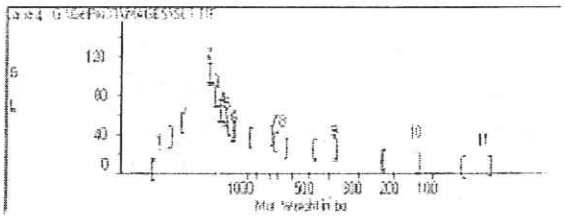
Fig. (V): Electrophoretic pattern in control (lane 1) - dependence "group II a" (lane 2) - withdrawal "group II b" (lane 3) -clonidine for 2 weeks during withdrawal "group II c" (lane 4) & clonidine for 4 weeks during withdrawal "group II d" (lane 5).



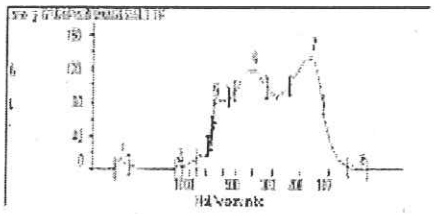
Bichart of lane (3)



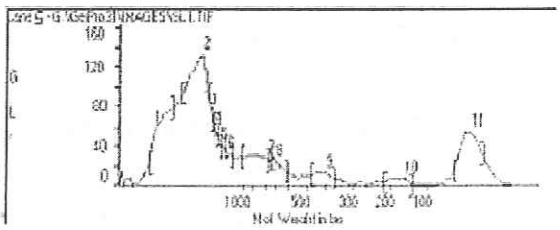
Bichart of lane (1)



Bichart of lane (4)



Bichart of lane (2)



Bichart of lane (5)

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تغيرات الخلايا النووى الناتجة من سوء إستخدام عقار الكلونازيبام بمخ جرذان التجارب البيضاء ودور عقار الكلونيدين أثناء فترة الانسحاب

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تعتبر مادة الكلونازيبام إحدى مشتقات مجموعة البنزوديازيبام، والتى يتم وصفها لعلاج الكثير من الأمراض العصبية والنفسية هى الأكثر إدماناً بين المرضى فضلاً عن المدمنين من غير المرضى، ولقد تم دراسة مادة الكلونازيبام كمادة إدمانية للوقوف على أثرها التدميرى على مخ الجرذان بعد إدمانها، وأيضاً تم تقييم أثر مادة الكلونيدين من حيث آثارها الواقية فى مرحلة الانسحاب من الكلونازيبام، وقد تم حفظ عشرة من الجرذان البيضاء كمجموعة ضابطة سلبية (المجموعة الأولى) دون أى عقار فى ظروف قياسية لمدة شهر قبل ذبحها، وقد تم إحداث الإدمان بمادة الكلونازيبام فى عدد أربعين جرذاً بإعطائها جرعات متدرجة فى الزيادة لمدة شهر (المجموعة الثانية) ثم قسمت لأربعة مجموعات متساوية.

المجموعة الثانية أ : (مجموعة إدمان الكلونازيبام) عشرة جرذان ذبحت فى نهاية شهر الإدمان بالكلونازيبام.

المجموعة الثانية ب : (مجموعة الانسحاب) عشرة جرذان تركت بدون الكلونازيبام لمدة شهر بعد إدمانها ثم ذبحت.

المجموعة الثانية ج : (مجموعة علاج كلونيدين لمدة أسبوعين) عشرة جرذان أعطيت عقار الكلونيدين لمدة أسبوعين أثناء فترة الانسحاب من الكلونازيبام.

المجموعة الثانية د : (مجموعة علاج كلونيدين لمدة أربعة أسابيع) : عشرة جرذان تلقت عقار الكلونيدين لمدة أربعة أسابيع أثناء فترة الانسحاب من الكلونازيبام.

المجموعة الثالثة (مجموعة ضابطة موجبة للكلونيدين) : عشرة جرذان تتلقى الكلونيدين لمدة شهر.

وقد تم ذبح جرذان المجموعات السابقة فى نهاية الفترات المحددة لكل مجموعة.

وقد بينت الدراسة أن التكسير المبرمج المنتظم للحامض النووى (DNA degradation; apoptosis) فى أنسجة مخ الجرذان

البيضاء المدمنة للكلونازيبام (المجموعة الثانية أ) ذو دلالة إحصائية عالية مقارنة بالمجموعة الضابطة وذلك باستخدام تقنية الفصل الكهربى الجيلى وتحليل النتائج ببرنامج Pro-analyzer. كما تبين أيضاً حدوث التكسير المرحلى المبرمج للحمض النووى Apoptosis فى المجموعة الإنسحابية (الثانية ب) وإن كانت أقل من مجموعة الإدمان إلا أنها كانت أعلى من المجموعة الضابطة وذو دلالة إحصائية عالية، كما وجد أيضاً أن مادة الكلونيدين (المجموعة الثانية ج) لمدة أسبوعين أقل فى درجة تكسر الحمض النووى عن المجموعة الإدمانية والانسحابية بفارق ذو دلالة إحصائية، كما وجد أن التكسر فى الحمض النووى فى المجموعة الثانية د بعد علاج الكلونيدين مدة أربعة أسابيع أقل بكثير عن المجموعات الإدمانية والانسحابية والانسحابية مع الكلونيدين لمدة أسبوعين، وقد وجد أن التغيرات السابقة قد اتفقت مع التغيرات الهستوباثولوجية والهستوكيميائية.

ومن هذه الدراسة يجب توخى الحرص عند وصف عقار يحتوى على الكلونازيبام للمرضى مع مراقبتهم لإمكانية تحولهم لمدمنين لهذا العقار، كما تبين أيضاً أن لمدة العلاج بالكلونيدين تأثير واق أثناء فترة الانسحاب من مادة الكلونازيبام.

وننصح بتجربته على مرضى من البشر أو مدمنين أثناء فترة الانسحاب من مادة الكلونازيبام.