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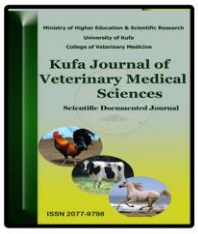

The neurobehavioral and withdrawal effects of diazepam in adult male rats

Mohammed Jawad kadhim Falah Muosa Kadhim AL-Rekabi
Duriad Abdulhadi Abass
College of vet-medicine, university of Baghdad
E-mail ; fab20062003@yahoo.com

Abstract:

The study conducted to evaluate the neurobehavioral effects and dependence in adult male rats which showed significant decrease ($p<0.05$) in exploration, vestibular system (increasing time of rotating on negative geotaxis apparatus), decrease cognition, short term memory, locomotors activity (decrease time of rotating on Rota rod apparatus), where these results was confirmed by histopathological changes of brain which represent by congestion, perivascular edema, focal gliosis and mononuclear cells infiltrations in groups treated with diazepam. The results of withdrawal study after one week of stop dosing of diazepam treatment showed signs of addiction represented by hyperactivity, restlessness, diarrhea and goose flesh in dose dependent manner.

Key word; neurobehavioral , withdrawal. Diazepam , male rat.

محمد جواد كاظم فلاح موسى كاظم الركابي

دريد عبد الهادي عباس

جامعة بغداد\كلية الطب البيطري

الخلاصة:

صممت هذه الدراسة لتقييم تاثيرات السلوك العصبي والادمان لعقار الديازبم في ذكور الجرذان البالغه حيث اظهرت النتائج نقصاناً معنوياً على مستوى ($P<0.05$) في الحركه الموضعيه والاستكشافيه وخلل في وظائف الجهاز الدهليزي(زيادة الوقت اللازم للاستدارة لتجنب الانحدار) وتأثر الذاكرة قصيرة الامد وخلل في درجة اليقظه والادراك وفي التوافق العصبي العضلي(نقصان فترة البقاء على العصا الدوارة) حيث تم تأكيد هذه النتائج من خلال التغيرات النسجية في ادمغة الجرذان المعالجة بالعقار تمثلت في وجود احقان ووذمه بين الوعائية و انسلال خلايا احادية النواة ودباق بؤري معتمدة على الجرعة ومدة الاعطاء. وكانت نتائج تأثير انسحاب عقار الديازبم بعد توقف الاعطاء لمدة اسبوع ظهور علامات الادمان متمثله في فرط النشاط وهيجان واسهال ولحم الوز في الجرذان معتمدة على الجرعة ومدة الاعطاء.

Introduction:

Diazepam is a benzodiazepine that exerts anxiolytic, sedative, muscle relaxant, anticonvulsant and amnestic effects. Most of these effects are thought to be a result from a facilitation

of the action of GABA, an inhibitory neurotransmitter in the central nervous system [1]. Some of studies refer to reduced exploratory activities in rats after long term administration of diazepam may be the consequence of neuronal degeneration and cerebellar damage which might have consequently impaired cerebellar functions that partly manifested as suppressed exploratory activities in the treated rats **[2].** Diazepam, as with other benzodiazepine drugs, can cause tolerance, physical dependence [3]. Therefore the study was design to assessment the neurobehavioral and withdrawal effects of diazepam in adult male rats.

Materials and methods:

Thirty adult male albino Wister rats procured from college of medicine of Baghdad university and raised in the animal house (college of veterinary medicine, university of Baghdad) in the air condition room at the temperature 23±2, and fed standard pellet food. The animal divided equally into three groups, the first group T1 had dosed 0.1mg/kg.BW diazepam orally through stomach tube, half number of animal discontinued after two weeks while the remain animal were continued dosing till the end of one month, the animals of second group T2 had dosed diazepam 0.6mg/kg.BW orally in the same manner of the first group. Third group had dosed distilled water and considered control group.

1-Neurobehavioral tests include:-

A-Head pocking:

This test determines the degree of cognitive function of animal **[4]**.

B. Rota Road

This test reflects neuromuscular coordination or ataxia **[4]**

C. Passive avoidance

This test performed to evaluate the effect of introduced substance on short term memory of animal **[5]** by studying the external shocking stimulus on the animal and calculate the number of shocks which are needed to return to the safe zone.

D. Negative Geotaxis

This test reflects vestibular function, neuromotor performance and coordination **[5].**

E. Open field

The test evaluates the general locomotor activity, exploration, rearing and also including frequency of defecation and urination (Autonomic nervous impressed), rat placed on the center of arena of open field apparatus and the number of squares crossed, number of rearing, number of fecal boluses and urine pools during 3 minutes were counting. Arena was cleaned after each trying by cotton without alcohol **[6].**

F. Arousal

It could be indicate for attention of animal which influence consciousness in open field **[7].**

G. Touch response

It depends on response of animal to external sensitization which is performe by sensitizing the animal with solid object in near to the nose then record the animal response grade **[8].**

H. Swimming rank test

This test reflects the integration of brain function by monitoring each animal for (5-10) seconds for swimming in the pool containing warm water 30ºC**.**

Withdrawal assessment:

At the end of dosing, at day (14, 30) of treatment, the drug had discontinued for one week and the animal observed for withdrawal signs, The signs recorded according to withdrawal classification depending on clinical observation of animal and have been scored (0. 1, 2) according to its severity and the total score had calculated and classified as: 1-5 mild, 6-10 moderate, and 10-15 severe, table (1)[10]:

withdrawal Signs	
Diarrhea	Dilated pupils
Hyperactivity	Hypertension
Restlessness	Lacrimation
Goose flesh	Muscle cramps
Insomnia	Bowel sound

Statistical analysis:

The data was subjected to SPSS version 13.00 where One way ANOVA was used to assess significances at $p<0.05$ and less score differences multiple range test is carried out to compare between groups [10].

Results and discussions:

Both treated groups (T1 and T2) showed no significant differences of the all studied neurobehavioral parameters at the zero time in comparison to the animal of the control one. The animals of group had treated with diazepam 0.1mg/kg.BW of diazepam orally showed significant decrease $p<0.05$ in the number of head pocking, square crossed, arousal, fecal bolus, negative geotaxis and swimming rank only after four weeks of treatment in comparison to control one, tables (2,3,6,7,11,12), but they showed significant decrease $p<0.05$ in the number of rearing, touch response grade, number of shock needed for passive avoidance and time spent on the Rota rod after two and four week of treatment in comparison to the control one, tables (4,5,9,11). The urine pool frequency per three minute showed no significant differences between the animals of this group and the control one. The animals of group had treated with diazepam 0.6mg/kg.BW of diazepam orally showed significant increase in the number of fecal bolus and urine pools after four weeks only table (7,8), but they showed significant decrease $p<0.05$ in the number of rearing, head pocking, square crossed, arousal, negative geotaxis, swimming rank, touch response grade, number of shock needed for passive avoidance and time spent on the Rota rod after two and four weeks of treatment in comparison to the control one, tables (4,5,9,2,3,6,11,12,10).

Table (2): Head pocking test (number of face entrance in pores 3/minutes) of adult male rats dosed orally with diazepam at different doses and periods

Period / Group	zero time mean ±S.E	Two weeks mean ±SE	Four weeks mean ±SE
Control	7.40±0.44 A a	6.90±0.48 A a	7.30±.0.36 A a
T1	6.90±0.62 A a	6.30±0.47 AB a	4.70±0.42 B b
T2	7.80±0.62 A a	5.50±0.30 B b	3.00±0.33 C c

LSD=1.35, Small letters represent different within group (p<0.05)
Capital letters represent different between groups (p<0.05)
T1: treated with diazepam 0.1mg/kg.BW, T2 treated with diazepam 0.6 mg/kg.BW, C: control dosed with B.W

Table (3): Open field (number of square crossed/ 3minute) in adult male rats dosed orally with diazepam at different doses and periods

Period / Group	zero time mean ±SE	Two weeks mean ±SE	Four weeks mean ±SE
Control	40.20±1.88 A a	38.00±3.56 A a	38.60±0.92 A a
T1	40.40±1.56 A a	35.20±0.91 AB b	32.60±1.15 B b
T2	39.80±1.80 A a	32.40 ±1.20 B b	22.80 ±0.66 C c

LSD=4.04 , Small letters represent different within group (p<0.05)
Capital letters represent different between groups (p<0.05)
T1: treated with diazepam 0.1mg/kg.BW, T2 treated with diazepam 0.6mg/kg.BW, C: control dosed with D.W

Table (4): Open field (number of rearing / 3minute) in adult male rats dosed orally with diazepam at different doses and periods

Period / Group	zero time mean ±SE	Two weeks mean ±SE	Four weeks mean ±SE
Control	8.80±0.66 A a	8.40±0.60 A a	7.80±0.73 A a
T1	8.40±0.40 A a	6.60±0.81 B b	3.60±0.60 B c
T2	7.80±0.48	5.40±0.50	3.00±0.44

	A a	B b	B c

LSD=1.67, Small letters represent different within group (p<0.05)
Capital letters represent different between groups (p<0.05)
T1: treated with diazepam 0.1mg/kg.BW, T2 treated with diazepam 0.6mg/kg.BW, C: control dosed with D.W

Table (5): Touch response test \ grade in adult male rats dosed orally with diazepam at different doses and periods.

Period / Group	zero time mean ±SE	Two weeks mean ±SE	Four weeks mean ±SE
Control	3.80±0.20 A a	3.89±0.20 A a	3.77±0.14 A a
T1	3.90 ±0.12 A a	2.70±0.24 B b	2.00±0.31 B c
T2	3.85±0.10 A a	2.20±0.20 C b	1.60±0.24 C c

LSD=0.49, Small letters represent different within group (p<0.05)
Capital letters represent different between groups (p<0.05)
T1: treated with diazepam 0.1mg/kg. BW, T2 treated with diazepam 0.6mg/kg.BW, C: control dosed with D.W

Table (6): Arousal test in adult male rats dosed orally with diazepam at different dose and periods.

Period / Group	zero time mean ±SE	Two weeks mean ±SE	Four weeks mean ±SE
Control	3.10±0.17 A a	2.90±0.10 A a	3.22±0.23 A a
T1	3.01 ±0.21 A a	2.70±0.15 A a	2.40±0.24 B a
T2	2.90±0.17 A a	2.10±0.17 B b	2.00±0.31 B b

LSD=0.68, Small letters represent different within group (p<0.05)
Capital letters represent different between groups (p<0.05)
T1: treated with diazepam 0.1mg/kg. BW, T2 treated with diazepam 0.6mg/kg.BW, C: control dosed with D.W

Table (7): Number of fecal bolus/ 3minute in adult male rats dosed orally with diazepam at different doses and periods

Period / Group	zero time mean ±SE	Two weeks mean ±SE	Four weeks mean ±SE
Control	2.70±0.36 A a	2.50±0.29 A a	2.20±0.37 A a
T1	2.80±0.45 A a	3.10±0.56 A ab	3.95±0.66 B b

T2	2.40±0.24 A a	3.20±0.27 A a	4.40±0.81 B b

LSD=1.11, Small letters represent different within group (p<0.05)
Capital letters represent different between groups (p<0.05)
T1: treated with diazepam 0.1mg/kg.BW, T2 treated with diazepam 0.6mg/kg.BW, C: control dosed with D.W

Table (8): Number of urine pools/ 3minute in adult male rats dosed orally with diazepam at different doses and periods

Period / Group	zero time mean ±SE	Two weeks mean ±SE	Four weeks mean ±SE
Control	2.30±0.30 A a	2.40±0.30 A a	2.60±0.24 A a
T1	2.30±0.33 A a	2.70±0.21 A a	3.20±0.20 AB a
T2	2.40±0.22 A a	2.90±0.23 A ab	3.80±0.58 B b

LSD=1.02, Small letters represent different within group (p<0.05)
Capital letters represent different between groups (p<0.05)
T1: treated with diazepam 0.1mg/kg.BW, T2 treated with diazepam 0.6mg/kgBW, C: control dosed with D.W

Table (9): Passive avoidance (number of shock to return to safe zone) in adult male rats dosed orally with diazepam at different doses and periods

Period / Group	Zero time mean ±SE	Two weeks mean ±SE	Four weeks mean ±SE
Control	1.20±0.2 A a	1.40±0.13 A a	1.60±0.24 A a
T1	1.40±0.24 A a	2.70±0.24 B b	2.44±0.20 B b
T2	1.40±0.40 A a	2.90±0.23 B b	3.80±0.58 C c

LSD=0.82, Small letters represent different within group (p<0.05)
Capital letters represent different between groups (p<0.05)
T1: treated with diazepam 0.1mg/kg.BW, T2 treated with diazepam 0.6mg/kg.BW, C: control dosed with D.W

Table (10): Rota rod performance test (rotating time spend on apparatus) \ second in adult male rats dosed orally with diazepam at different doses and periods.

Period / Group	zero time mean ±SE	Two weeks mean ±SE	Four weeks mean ±SE
Control	17.00±0.61 A a	19.80±1.24 A a	17.40±1.24 A a
T1	18.00±0.89 A a	14.60±0.87 B b	8.60±1.02 B c

T2	18.60±0.96 A a	12.60±0.50 B b	4.40±0.58 C c

LSD: 3.09, Small letters represent different within group (p<0.05)
Capital letters represent different between groups (p<0.05)
T1: treated with diazepam 0.1mg/kg.BW, T2 treated with diazepam 0.6mg/kg.BW, C: control dosed with D.W

Table (11): Negative geotaxis test (60 second) in adult male rats dosed orally with diazepam at different doses and periods.

Periods / Groups	zero time mean ±SE	Two weeks mean ±SE	Four weeks mean ±SE
Control	2.40±0.50 A a	2.60±0.50 A a	2.80±0.58 A a
T1	2.60±0.50 A a	4.20±0.66 A a	6.80±1.11 B b
T2	2.20±0.37 A a	7.40±0.74 B b	11.40±1.86 C c

LSD=1.87, Small letters represent different within group (p<0.05)
Capital letters represent different between groups (p<0.05)
T1: treated with diazepam 0.1mg/kg.BW, T2 treated with diazepam 0.6mg/kg.BW, C: control dosed with D.W

Table (12): Swimming rank test (grade) of adult male rats dosed orally with diazepam at different dose and periods.

Period / Group	zero time mean ±S.E	Two weeks mean ±S.E	Four weeks mean ±S.E
Control	3.80±0.13 A a	3.80±0.20 A a	3.60±0.40 A a
T1	3.81±0.18 A a	3.20±0.20 A b	2.50±0.20 B c
T2	3.77±0.12 A a	2.20±0.37 B b	1.60±0.24 B c

LSD= 0.69, Small letters represent different within group (p<0.05)
Capital letters represent different between groups (p<0.05)
T1: treated with diazepam 0.1mg/kg.BW, T2 treated with diazepam 0.6mg/kg.BW, C: control dosed with D.W

The results of neurobehavioral testes in adult male rats dosed orally with diazepam in different periods and doses showed significant decrease in all of exploratory activities, locomotors, memory, cognition and swimming performance, as well as there were decrease in poking, Rota rod time, swimming rank test, square crossed, touch response in dose- period related manner.

The neurobehavioral parameters changes in this study may be attributed to the effect of diazepam on dopamine and or another catecholamine or other neurotransmitters of central nervous system like serotonin, glutamate and acetylcholine (Ach) because of the ability

of diazepam to penetrate Blood Brian Barrier (BBB), and cause pathological changes such (perivascular edema, focal proliferation of microglia cells and congestion) after long term diazepam administration, that's may be affect release of neurotransmitter and may be affect on neurobehavioral in dose and period dependent manner. Catecholamines including dopamine and nor epinephrine are the principle neurotransmitters that mediate a variety of the central nervous system functions such as motor, control, cognition, emotion, processing and endocrine' dysfunction in Catecholamines neurotransmitters are implicated in some neurological and neuropsychiatric disorder reported by [11]. In general, the decrease of dopaminergic neurotransmission leads to decrease in locomotor activity, the results showed decrease in the time of rotating on rod which demonstrates to impairment of locomotors activity. Diazepam negatively affects motor coordination and balance and produce myorelaxation, when it act on α1 GABAA receptors mediate to ataxia and indirectly contribute to myorelaxation in rats.

However, different terminal region and different receptors subtype do not contributed in the same way [12] also the central dopaminergic system is important for regulation of variety of processes including cognitive, memory, attention and problem solving, also dopamine is strongly implicated in higher order cognitive functioning [13].

Diazepam significantly depressed the release of monoamine neurotransmitters caused by elevation of potassium (K^+) in extracellular as a result of repolarizing effect of diazepam on GABA receptor. Where it have depressive effects on the releases of nor adrenaline (NA) and dopamine (DA) which play a role in sending of signals from nerve cells to other nerve cells and other effect organs [14]. The effects of diazepam in neurobehaviorals observe throughout their effects in neurotransmitters such as dopamine, nor adrenaline and acetylcholine.

We hypnotized the increasing urine and fecal bolus in both treated due to interfering of diazepam with adrenaline (decrease releasing) which lead to parasympathetic system (acetylcholine) effect to overcome the sympathetic system in GIT. Also the decrease in neuromuscular performance, coordination, affection of short term memory and blood integrity in animals treated with diazepam in both young and young rats may be due to the disturbance in the releasing of monoamines neurotransmitter like dopamine, serotonin, and also Ach releasing.

2-Withdrawal assessments:

The results of withdrawal effects of adult male rats dosed with diazepam at different doses and periods showed appearances of mild withdrawal signs after two and four weeks of both treated groups except for T2 after four weeks that showed appearance of moderate withdrawal signs. The numbers represented the mean of grade (0, 1, 2) according to withdrawal classification by [10] and the severity of signs measuring throughout scoring the mean of signs grade and classed to be mild (1-5), moderate (6-10) and severe (10-15), table (13).

Withdrawal effects/grade of adult male rats dosed orally with diazepam at different doses and periods.

Group/periods / Symptom	T1		T2	
	2Weeks	4Weeks	2Weeks	4Weeks
Goose flesh	0	0.50	0.60	1.80
Hyperactivity	0	1	1	2
Restlessness	0.80	1	1	2
Diarrhea	0	0.60	1	1.80
Total	0.80	2.6	3.60	7.80
Severity	Mild	Mild	Mild	Moderate

T1: treated with diazepam 0.1mg/kg.BW, T2 treated with diazepam 0.6mg/kg.BW

The results of withdrawal assessments in adult male rats dosed orally with diazepam at different periods and doses showed withdrawal signs after two and four weeks ranged from mild to moderates signs in dose-period dependent manners represent as (goose flesh, hyperactivity, restlessness and diarrhea).

Exposure to diazepam causes neural adaptations that counteract the drug's effects, leading to tolerance and dependence [15]. Despite taking a constant therapeutic dose, long-term use of benzodiazepines may lead to the emergence of withdrawal-like symptoms [16].

The neuroadaptive processes involved in tolerance, dependence, and withdrawal mechanisms implicate both the GABAergic and the glutamatergic systems [15]. Gamma-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter of the central nervous system GABA mediates the influx of chloride ions through ligand gated chloride channels called $GABA_A$ receptors. When chloride enters the nerve cell, the cell membrane potential hyperpolarizes thereby inhibiting depolarization, or reduction in the firing rate of the post-synaptic nerve cell [17]. Diazepam potentiates the action of GABA [18]. Thereby increase the frequency of the GABA-gated chloride channel opening in the presence of GABA [19]. When potentiation is sustained by long-term use, neuroadaptations occur, which result in decreased GABAergic response. What is certain, is that surface $GABA_A$ receptor protein levels are altered in response to diazepam exposure as is receptor turnover rate [20].

Other hypotheses including changes in the receptor conformation, changes in turnover, recycling, or production rates, degree of phosphorylation and receptor gene expression, subunit composition, decreased coupling mechanisms between the GABA and benzodiazepine site, decrease in GABA production, and compensatory mechanism increased gutamatergic activity [20].

Diazepam is cleared from the brain; these neuroadaptations are unmasked leading to unopposed excitability of the neuron [21]. Glutamate is the most abundant excitatory neurotransmitter in the vertebrate nervous system [22]. Increased glutamate excitatory activity during withdrawal may lead to sensitization of the CNS and appearance of excitatory signs [23].

Histopathological Changes:

The brain of adult male rats' dosed orally with diazepam 0.1mg/kg daily for two weeks showed no pathological changes, Figure (1). After four weeks showed perivascular edema of congested blood vessels and around glial cells, Figure (2), the brain of adult male rat's brain oral dosed with diazepam 0.6mg/kg daily for two weeks showed no clear pathological changes, Figure (3). but after four weeks of treatments showed focal gliosis characterized by focal proliferation of microglia cells and edema around some them Figure (4,5).

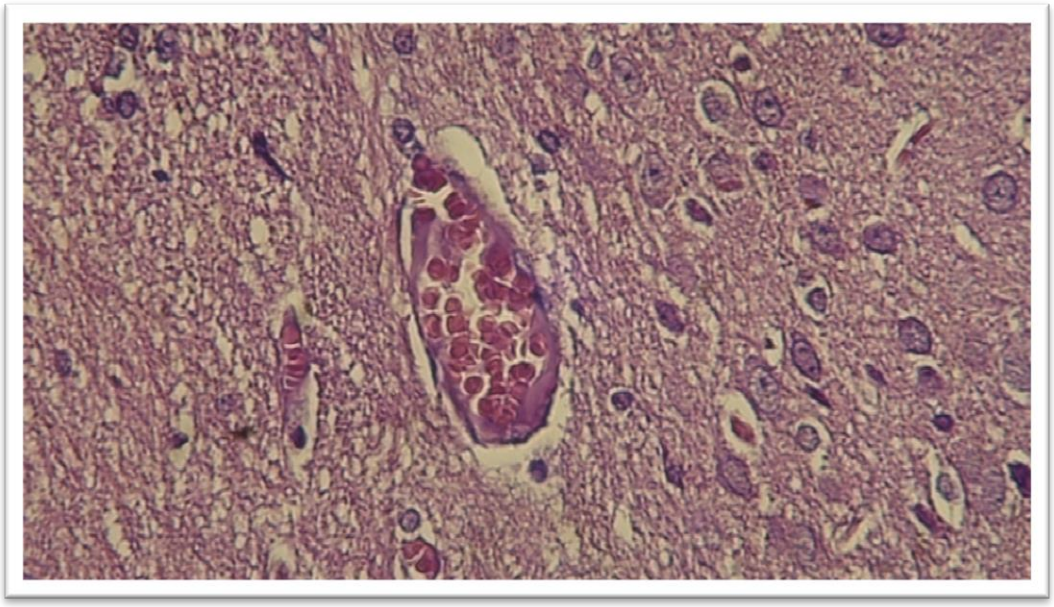

Figure (1): brain of adult male rat dosed orally with diazepam 0.1mg/kg daily for two weeks showed no clear pathological changes. H and E. 40X

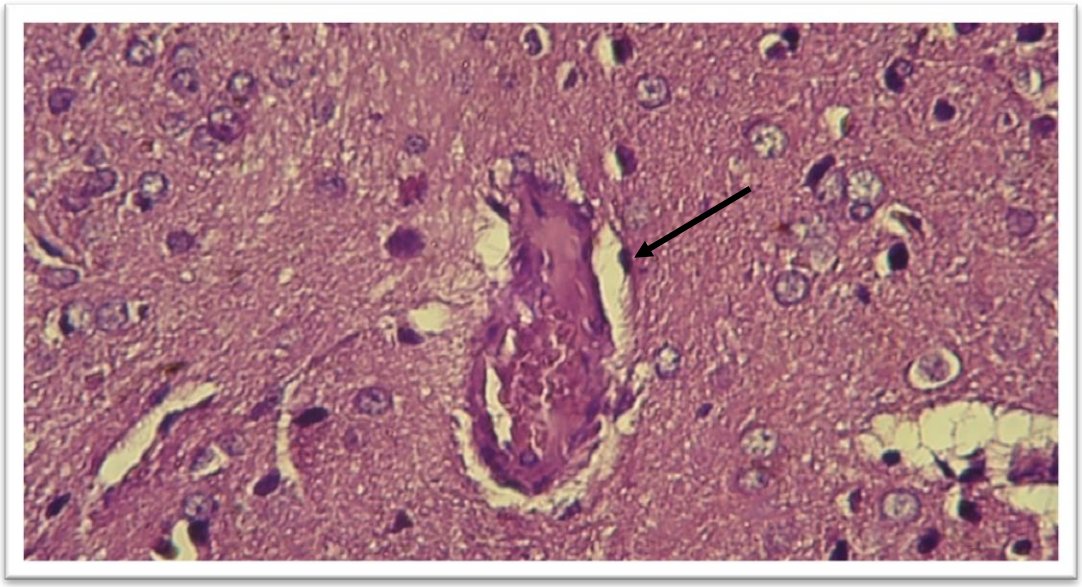

Figure (2): brain of adult male rat dosed orally with diazepam 0.1mg/kg daily for four weeks showed perivascular edema of congested blood vessels and around glial cells. H and E. 40X

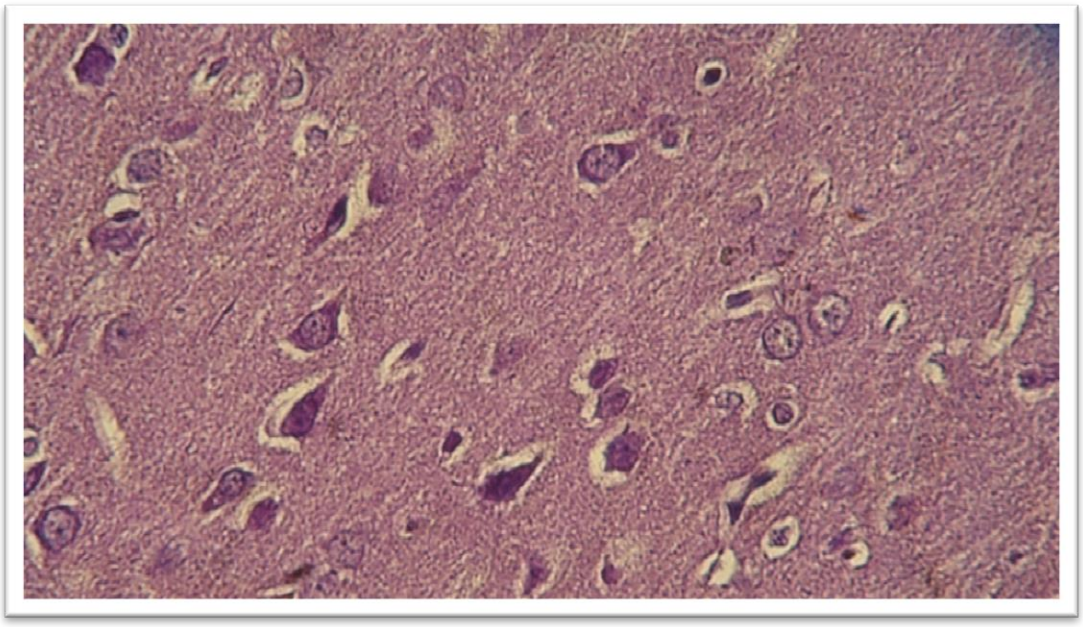

Figure (3):): brain of adult male rat dosed orally with diazepam 0.6mg/kg daily for two weeks showed no clear pathological changes. . H and E. 40X

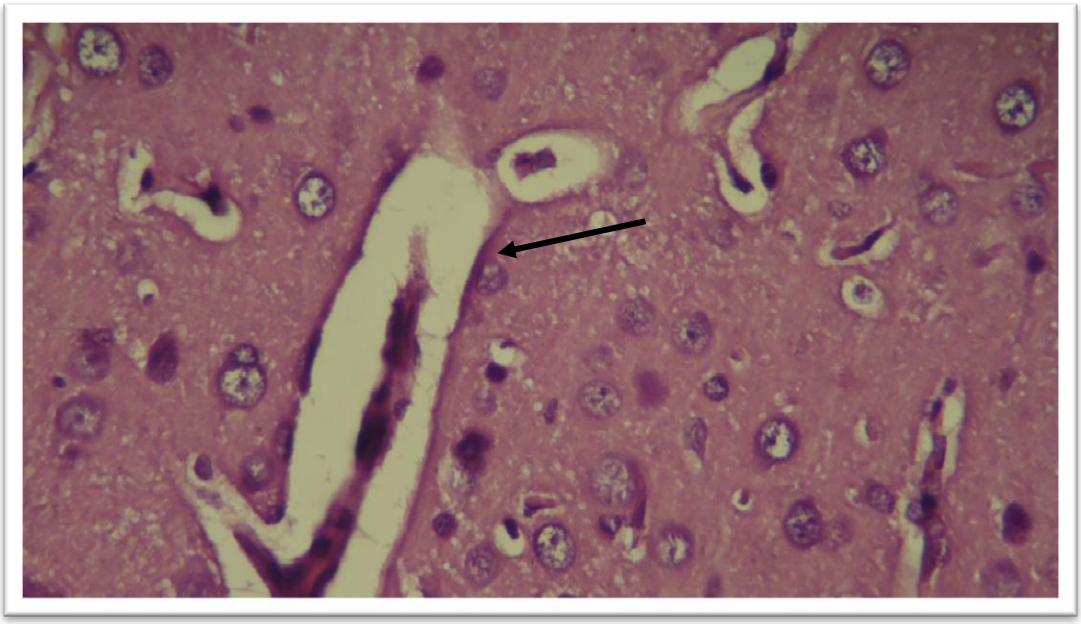

Figure (4):): brain of adult male rat dosed orally with diazepam 0.6mg/kg daily for four weeks showed perivascular edema and around glia cells. H and E. 40X

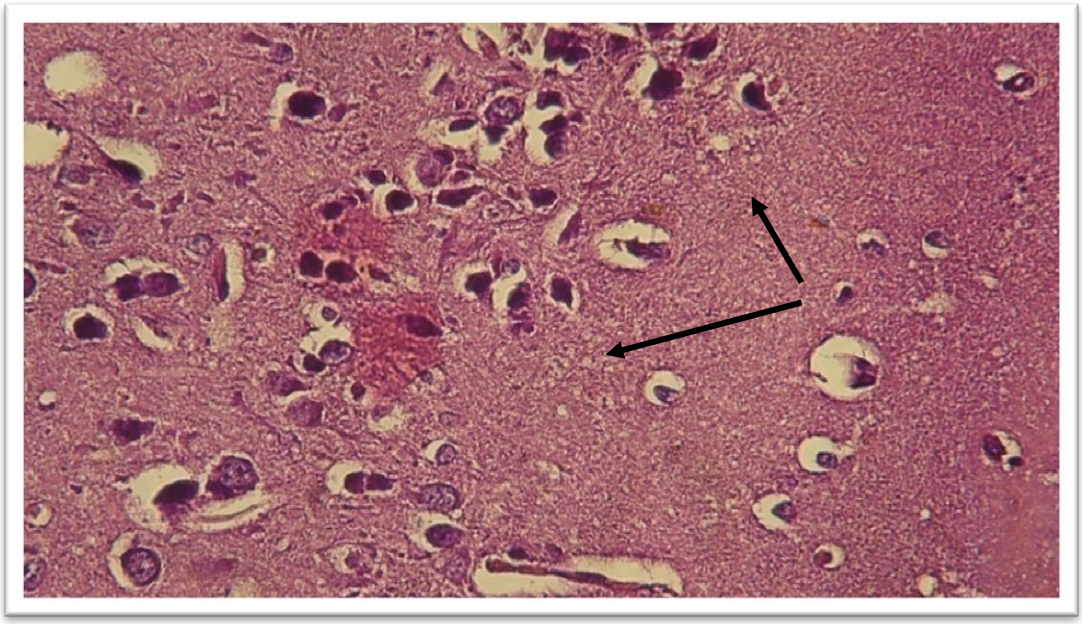

Figure (5): brain of adult male rat dosed orally with diazepam 0.6mg/kg daily for four weeks showed focal gliosis characterized by focal proliferation of microglia cells and edema around some them. H and E. 40X

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