

The Histologic Effect of Baclofen in Rat's brain: An Experimental Study

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Abstract

Background: Baclofen is the only clinically available metabotropic GABAB receptor agonist. Several reports has been discussed the efficacy of baclofen but the available data on its effect of the behavior and brains' histology are still limited.

Objectives: This work aims to assess the effects of long term exposure to baclofen (8weeks) on behavior and brains' histology in rats

Methods: Analysis of data of Twenty five healthy male Albino rats were performed. Rats weighing about 200–210 g with mean age of 65 days were randomly selected and categorized into three groups: Group 1(LD50 group): included five rats that gavaged with single dose of baclofen to test the LD50 of drug (145mg per Kg) .Group 2. (control group):included eight rats were gavaged with distilled water for 2 months. Group 3.(baclofen group): included 12 rats gavaged with 15mg/kg of baclofen (1/10th LD50) for 2 month. Behavioral Tests were conducted on all animals in group 2 and 3 including: Open-field test ,Open -field habituation task ,and object recognition(Novel) to examine the influence of drug on the cognition of rats. All animals were euthanized with ether after 24hours of last day of treatment. Brains were excised and collected from rats of group 2 and 3.Fixation of tissues was done using buffered solution of ten percent of formalin for twenty four hours with paraffin wax embedding. Sectioning and staining by hematoxylin and eosin protocol (H&E) and prepared to be examine histologically. To differentiate between the data of group 2 and 3 ,statistical analysis was performed by 1- way analysis of variance (ANOVA) .Least significant difference test with Duncan test was used . Value of P of equal or less than 0.05 means the difference is significant .

Results: This study revealed that the rats of baclofen group traveled a greater total distance(in both sessions) when compared with those of control indicating their hyperactivity. The zone specific analysis showed that the rats that were administered with baclofen consumed markedly greater time nearby the walls in the peripheral

part in the arena throughout the first and 2nd 15- min session in comparison with those of control group indicating the generalized hyperactivity and exploration. Rats of control group preferred the novel object in comparison with those of baclofen group. However there is no marked difference in the index of discrimination among rats of group 2 and 3. Histological sections of rats' brains in group that were administered with 15mg/kg of baclofen for 2 month(baclofen group) revealed a diminished thickness in the pyramidal cells' layer, irregular faintly stained cells with shrinkage in the some pyramidal nerve cells, dark staining cytoplasm and losing the characteristics of their nuclei.

Conclusions:Chronic administration with baclofen (15mg/kg/day/2months) induces changes in the brain histology in rats, however, baclofen has no adverse effect on the behavior. To the best of our information, our work is the 1st that focus on the influence of baclofen on both the behavior and brains' histology. Our data suggesting that baclofen may have clinical utility.

Keywords:_rat, brain, histopathology, baclofen, behavior.

التأثير النسيجي للباكولوفين لدى دماغ الجرذ :دراسة تجريبية

. الخلاصة

الخلفية: إن الباكلوفين هو المحفز المتوفر سريريا لمستقبلات كابا بيتا. يضع من التقارير ناقشت الكفاءة للباكولوفين ولكن البيانات المتوفرة لتأثيره على السلوك ونسيج الدماغ لاتزال محدودة.

الاهداف: يهدف هذا العمل الى دراسة تأثيرات الاعطاء المزمن للباكولوفين لمدة ثمانية اسابيع على السلوك ونسيج الدماغ في الجرذان.

طرق العمل: تم تحليل البيانات لخمس وعشرون جرذا امهقا من الذكور الاصحاء. وزن الجرذان يتراوح بين 200- 210 غراما بمعدل عمر 65 يوما تم اختيارهم عشوائيا وقسموا الى 3 مجاميع: المجموعة الاولى(الجرعة المميئة للنصف) شملت خمس جرذان تم تجريعهم بجرعة وحيدة من الباكلوفين لفحص تأثير الجرعة المميئة للنصف من الدواء (145مغ لكل كغم). المجموعة الثانية (مجموعة السيطرة)تشمل ثمانى جرذان تم تجريعهم بالماء المقطر لمدة شهرين.المجموعة الثالثة (مجموعة الباكلوفين) تضمنت اثني عشر جرذا جرعا ب 15 مغم لكل كغم من الباكلوفين(10|1من الجرعة المميئة للنصف)لمدة شهرين.تم اجراء فحص السلوك لكل الحيوانات في المجموعة الثانية والثالثة ويشمل ذلك: فحص الحقل المفتوح, فحص الحقل المفتوح لمهمة التعود,

فحص تمييز الجسم المستجد لفحص تأثير الدواء على الإدراك في الحيوانات. تم إجراء القتل الرحيم باللايثر بعد 24 ساعة من آخر يوم من العلاج. تم استئصال الأدمغة وجمعهم من المجموعة الثانية والثالثة وتثبيتهم بمحلول الفورمالين المتعادل بتركيز 10% لمدة 24 ساعة ووضعهم بشمع البارافين ثم تم قصهم وصبغهم بالهيماتوكسيلين إيوسين للفحص النسيجي. تم تحليل الاختلاف بين المجموعتين التجريبيتين احصائيا بواسطة تحليل المتغيرات ذو الطريق الواحد متبعا بالفارق المعنوي الأصغر واختبار دنكن حيث كان مستوى قيمة ب يعتبر معنوي عندما تكون اقل من 0.05.

النتائج: أظهرت هذه الدراسة ان الجرذان لدى مجموعة الباكلوفين عبروا مسافة اكبر في كلا الشوطين بالمقارنة مع مجموعة السيطرة مما يدل على نشاطهم العالي. ان التحليل الخاص بالمنطقة أظهر ان الجرذان المعاملين بالباكلوفين قضوا وقتا اطول قرب الجدران في المنطقة الخارجية في الساحة خلال الشوط الاول والثاني الذي استغرق كل منهما 15 دقيقة بالمقارنة مع مجموعة السيطرة مما دل على النشاط العالي والاستكشاف. ان جرذان مجموعة السيطرة فضلوا الجسم الجديد بالمقارنة مع اولئك العائدين لمجموعة الباكلوفين. ومه هذا كان الاختلاف في معيار التمييز بين المجموعتين غير معنوي. ان فحص النسيج الدماغي للجرذان الذين جرعوا بالباكلوفين (15مغم لكل كغم) لشهرين -مجموعة الباكلوفين لوحظ فيه نقصان في ثخن الخلايا الهرمية، ووجود خلايا غير منتظمة وذات صبغة فاتحة اللون مع خلايا هرمية عصبية منكمشة مع سايتوبلازم غامق ونقص في تفاصيل النواة.

الاستنتاجات: ان الاعطاء المزمن بالباكلوفين (15مغم لكل كغم|يوم إشرين) أحدث تغييرات في نسيج الدماغ لدى الجرذان مع العلم انه لم تكن هناك تأثيرات عكسية ملحوظة على سلوكهم. حسب معلوماتنا هذا اول بحث حاول دراسة تأثير الباكلوفين على كلا السلوك ونسجية الدماغ. نتائجا تقترح امكانية الاستفادة من الباكلوفين سريريا.

الكلمات المفتاحية: الجرذ، الدماغ، النسيجية المرضية، الباكلوفين، السلوك

INTRODUCTION

The receptors of Gamma-aminobutyric acid B – GABAB- acts metabotropically and chiefly to inhibit central nervous system (CNS) of mammals.

Beta-chlorophenyl – GABA (baclofen, Lioresal) is the only clinically obtainable GABA beta receptor agonist and it is used as the first-line treatment for rigid skeletal muscles and spasticity beyond traumatic

injured spinal cord, cerebral palsy, multiple sclerosis, and amyotrophic lateral sclerosis⁽¹⁾ beside its analgesic effects.

Little portion of this drug exclusively can crosses the blood–brain barrier, inducing the wanted effects⁽²⁾.

The generalized depression of CNS influences can results into fatigability, syncope, ataxic gate, low blood pressure , disturbances in psychology , and depression in both respiratory cardiovascular functions ⁽³⁾.

The receptors of Gamma-aminobutyric acid B are commonly scattered within the CNS. They are fully distributed in hippocampal region which acts crucially for memory and spatial (locative) learning⁽⁴⁾. There have been studies on GABAB receptor ligands and their participation in learning and memory processes⁽⁵⁾.

The effect of GABAB receptors agonists on cognitive processes using animal models have turned out to be rather inconsistent. Laboratory animal studies have reported that baclofen disrupts (in dose dependent manner), memory and learning in rodents after systemic intake (followed by behavioral testing e.g. avoidance condition test⁽⁶⁾ Morris water maze test⁽⁷⁾). Memory impairment has been described for oral administration of baclofen that leads to the incapability in remembering or recognition of familiar persons' names, locations and also for intrathecal administration^(8,9). Baclofen facilitates abstinence in chronic alcoholics, so that lead to an exponential raised its use at high doses and that may be the cause of mounting intoxication. In fact, baclofen toxicity is an under diagnosed emergency⁽¹⁰⁾. The precise pathways that stands behind the neuro-toxicity

after using of overdose of baclofen are still unclarified as the studies are unsatisfactory.

The rat model surely propagated humans' baclofen toxicities that is threatening their lives⁽¹¹⁾. Behavioral impairments, sleeping disturbances, and impairments in the activity of electroencephalograph are reported in the rats after administration with single / repeated dose of baclofen less than 20 mg/kg⁽¹²⁾.

On the other hand, a recent study showed that long term stimulation of GABA Beta receptors ameliorates the achievements on a multiple of cognitive and social duties that is shown to be diminished in 2 various mouse models of 16p11.2 deletion that were suggested to permit risks for autism spectrum disorder and intellectual disabilities which influences on about 3 in 10000 people⁽¹³⁾.

There is a paucity on the influence of long time exposure to baclofen on the cognitive function and brain structure in rats. The target of this research was to analyze the impact of prolonged administration with baclofen on behavior and brain histology in rats in order to discuss its contribution to the neurotoxicity –if –present.

MATERIALS and METHODS

Animals

Twenty five rats (Albino, male and healthy)were purchased from Animal House of Veterinary College, University of Mosul, Mosul, Northern Iraq.

Rats weighing about 200–210 g with mean age of 65 days. All of them were kept under well-controlled normal environmental and laboratory situations and animal facility and they were put separately in Animal House plastic cages (North Kent, England) measuring 47×34×18 cm filled from inside with wood chips⁽¹⁴⁾. Animals were prepared to acclimatize and left free for ten days before start any experiment ⁽¹⁵⁾, and will be supplied with free access of water *ad libitum* and packed optimized rodent chow ⁽¹⁶⁾. The procedures were conducted according the roles of the careful laboratory animals' usage with obtaining the approval of the Ethical Committee of College of Medicine ,Mosul University, choosing of few number of rats to gain the convenient results with avoiding of pain and stress. All experiments were conducted during the 12hours- light part ⁽¹⁷⁾. Rats were randomly selected and categorized into three groups⁽²⁾: **Group 1**(LD50 group) included five rats that gavaged with single dose of

baclofen to test the effect of LD50 of drug (145mg per Kg) ^(18,19,20). Observation of animals for any signs of manifestations of acute toxicities beside that of LD50 mortalities.

Group 2. (control group) included eight rats were kept quietly in a group without any stress, received distilled water for 2 month via gavage needle **Group 3.**(baclofen group) included 12 rats gavaged with 15mg/kg of baclofen (one tenth of LD50) in gradual raised doses for 2 months⁽²¹⁾.

Baclofen was obtained from some private pharmacies.

After drug dissolving in distilled water and it was gavaged orally via 24 gage needle . The rats were properly restrained to stabilize the head and kept in a vertical position. The gavage needle were appropriately fixed on Mantoux syringe and introduced through the side of the mouth, observed the roof of the mouth, and passed into the esophagus toward the stomach ,then the baclofen were injected ⁽²²⁾.

Each treated animal was monitored for unconcealed signs of toxicity for the next four hours, and mortality throughout the next 24hours. Then rats of groups 2 and 3 were submitted to the behavioral tests after 24hours of last day of treatment as the following:

Behavioral Test: It was performed in the following sequences :

1. Open-field test

This test is frequently utilized to evaluate anxiety and activity of locomotor system liking behaviors in experimental studies⁽²³⁾ . A box of wood with $40 \times 40 \times 40 \text{ cm}^3$ dimensions with fund divided to equal 25 squares was used to measure parameters throughout 5 minutes^(24,25).

2. Open -field habituation task

In the same previously mentioned box, this test was used during two sessions 15-min/ each , spaced 1–2 h apart. Animals were kept in the container of behavior and permitted for free explorer throughout each habituation's session. Sessions were registered and locomotor actions was observed. To analyze the data , the box was categorized into 2 zones: the 'inner' one (including the inner $30 \times 30 \text{ cm}^2$ central square) and an 'outer' one (the most peripheral area 5 cm from the margins). Computing the overall distance passes with the summation of distance of overall the course of each habituation's session.

3. The test of novel recognition of object

The test of novel recognition of object was done according to that of Leger et al, 2013⁽²⁶⁾. rats were accommodated to a $40 \times 40 \times 40 \text{ cm}^3$ box throughout 2×15 -minutes sessions, with space of 1–2 hr. in between. In between session, rats were brought back to the home cages. A day (24 hr.) beyond habituation, in the same box, rats were allow for exposure to two similar objects for 2×10 minutes exploring sessions with space of 1–2 h in between. Rats were demanded for the exploration of each object I a period of 10 s (overall of 20 s) in the 1st session to be enrolled in the consequent sessions. A day (24 hr.) after exploration of object, one object replacement with a novel one and the rats were permitted for the objects' exploration within 10 minutes. Recording of the time expended for exploration was performed throughout this exploring duration and was manifested by sniffing from 2 cm of each object or by direct touch of the objects. Excluding of the time expended on the object's top or climbing. Random assignment of both novel and familiar objects with the area of placement was done, by rat.

Calculation of index of discrimination using the following equation :

Time expended for exploration novel object /time expended for novel object's exploration +time expended for exploration of familiar object.

Histopathological Analysis:

At the last day of the designed work, all animals were euthanized with ether⁽¹⁴⁾after 24hours of last day of behavioral tests at the laboratory of Physiology , College of Dentistry, University of Mosul. All experimental works were conducted as the experimenter blind to protocols. Brains were excised collected from rats of all rats in a careful way during infectious precautions⁽¹⁷⁾and they were immediately weighed and examined macroscopically before they will be immersed in NaCl solution 0.9% for

RESULTS

All rats were survived all the experimental work .This study were analyzed the behavioral and histopathological data of 20 rats of group 2 and 3 blindly.

Locomotor activity :

Regarding the tests of behavior ,observations of rats' locomotor activity of the current study revealed that the rats that were administered with baclofen showed no marked

few seconds in order to get rid of superficial blood and fixation via solution of ten percent buffered formalin was done for 24 hr. and paraffin embedding. Sectioning and staining with hematoxylin and eosin protocol (H&E) to be examined histologically via light microscopical technique. Grading of tissue was recorded according to that described previously^(2,27).

Statistical analysis :Data were manifested as mean \pm SD , analysis of statistical difference among the experimental groups were done by 1-way analysis of variance (ANOVA) .Following that the least significant difference test and Duncan test was performed. P- Value of equal or less than 0.05 was considered as significant⁽²⁸⁾.

difference in their findings of open field locomotor activity in comparison with control group, however the rats which received baclofen exhibited some features of hyperactivity.

Open field habituation:

To examine the habituation in this condition, recording of the rats' activity of both groups across two 15-min recording sessions, spaced 1–2 h apart. During each habituation session, monitoring of activity inside a 40 \times 40

cm² container was done to identify the time expended in the 2 identified outer and inner zones of the field of behavior during sessions .

This study revealed that the rats that were administered with 15mg/kg of baclofen for 2 month(baclofen group) traveled a greater total distance(in both sessions) when compared with those of control indicating their hyperactivity (**Figure 1a**).

Across all treatments, rats spent equal period in context's exploration time throughout the 2nd recording session in comparison with the first session.

The zonal specified analyses showed that the rats that were administered with baclofen (baclofen group) expended markedly more time nearby the margins in the peripheral zone (of the arena) throughout the first and 2nd 15- minutes session in comparison with those of control group indicating the generalized hyperactivity and exploration(**Figure 1b**).

Object recognition:

This study examined the effect of treatment with baclofen via an object's recognition memories test as rats were permitted for the arena's exploration using 2 similar objects in paired 10-min sessions of exploring with a space of 1–2 hr. in between. After a day (24 hrs.), one of these objects was

substituted by a new object and an index of discrimination was estimated as (time expended in novel object's sniffings)/(overall time expended in the novel's sniffing +familial object) during a ten minutes investigating session .The rats of control group preferred the novel object in comparison with those of baclofen group. However , no marked differences in the index of discrimination between both groups(**Table 1**).

The current study revealed that the rats of both groups spent more time for the familiar object and they have not preferred for the novel object.

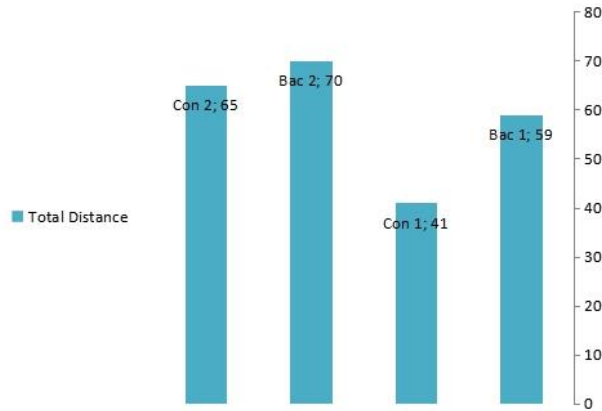
Histopathological Analysis:

This study revealed that the histopathological observations of sections of control animals showed normal structures of the brain including the polymorphic, pyramidal, and molecular layers. The neurons (pyramidal cells) appeared as big and has a shape of triangle as shown in **Figure 2a &b**.

When examining the brain tissue of the rats that were administered with 15mg/kg of baclofen for 2 month(baclofen group) ,there is diminishing of the pyramidal layer's thickness , irregular faintly stained cells with pyramidal nerve cells'

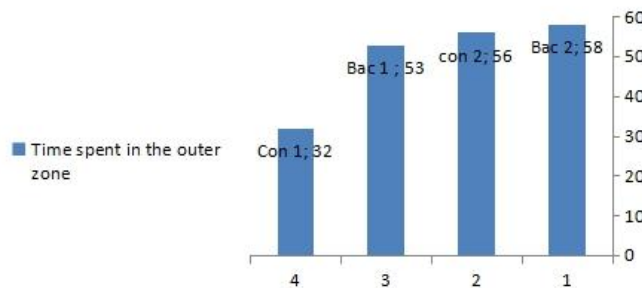
shrinkage with dark staining cytoplasm beside loosing details of nuclei (**Figure 3a,b,c**).

Figure 1a .A histogram of total Distance in both habituation sessions in control and baclofen group



*Con 1, the total distance in the first session in control rats ; Con 2, the total distance in the second session in control rats ; Bac 1 , the total distance in the first session in rats of baclofen group; Bac 2 ,the total distance in the second session in rats of control group.

Figure 1b .The Time spent in the outer zone in the first and second sessions of both groups



*Con 1, the time expended in the peripheral region in control rats in the first session; Con 2, the time expended in the peripheral region in control rats in the second session; Bac 1, the time expended in the peripheral region in the in rats of baclofen group in the first session ; Bac 2 , the time expended in the peripheral region in rats of baclofen group in the second session.

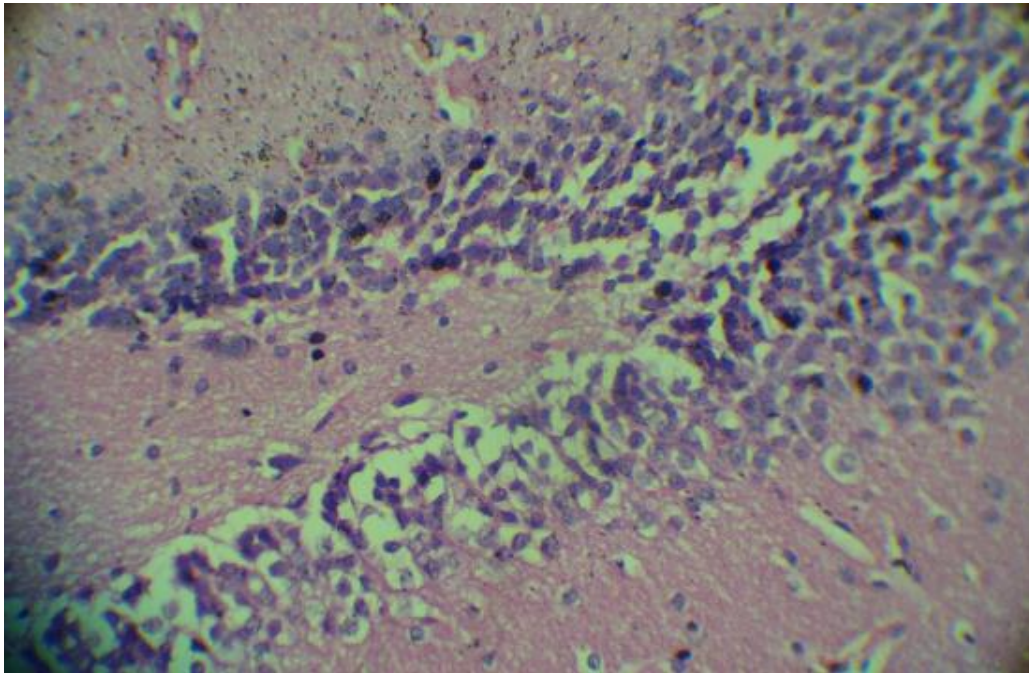


Figure 2b.A photomicrograph of a section from brain of control rat with normal architecture of the brain tissue. The big triangular pyramidal nerve cells are noticed(**H&E×400**).

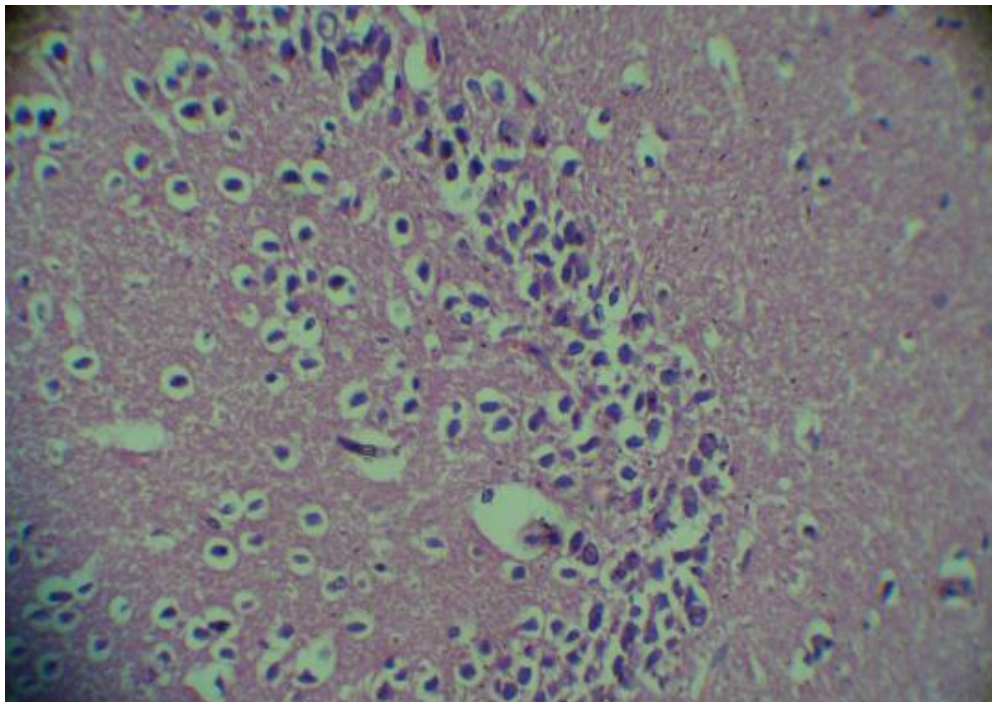


Figure 3a.A photomicrograph of a brain of rat that were administered with 15mg/kg of baclofen for 2 month (baclofen group) with decreased thickness of pyramidal cell layers.(**H&E×400**).

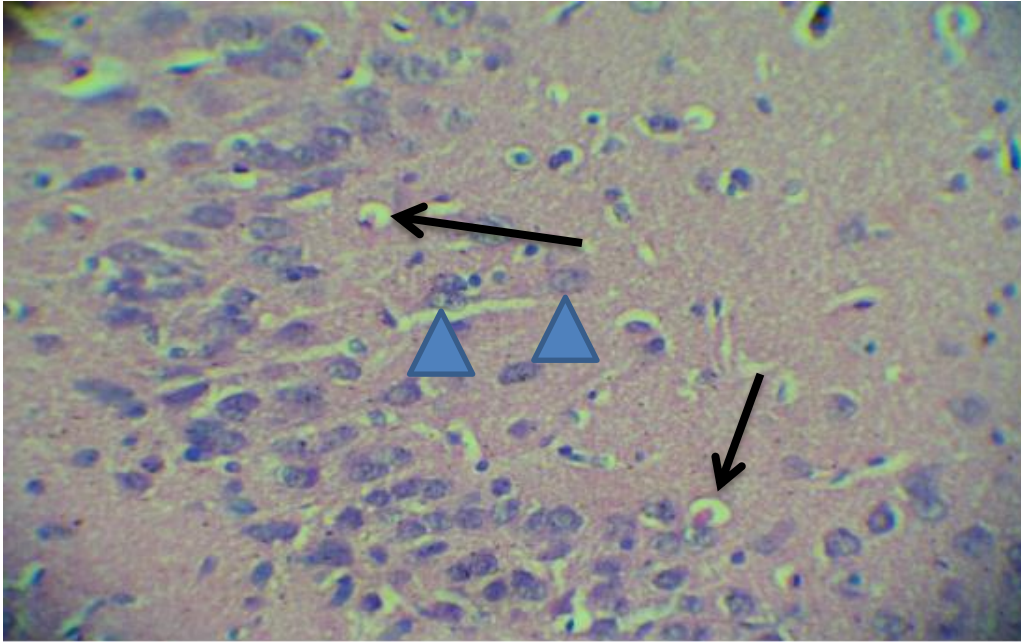


Figure 3b.A photomicrograph of a brain section from rat of baclofen group with diminished pyramidal layer's thickness, irregularly faint cells and other nerve cells with vesicles in nuclei and obvious nucleoli(arrow heads) and pyramidal neurons' shrinkage with a dark staining cytoplasm and losing the details of nuclei (arrow) (**H&E×400**).

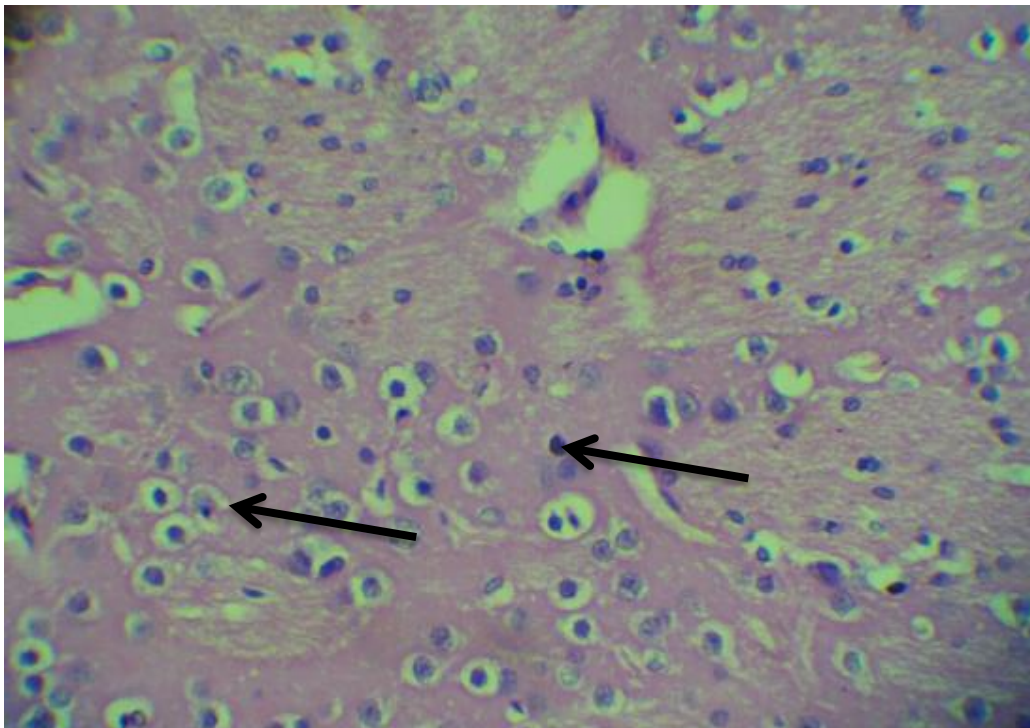


Figure 3c .A photomicrograph of a section of the rat's brain of baclofen group. Shrunken eosinophilic neurones with pyknotic nuclei(arrow)(**H&E×400**).

DISCUSSION

The current study analyzed the effect of chronic administration of rats with 15mg/kg of baclofen for 2 months on the behavior and the brain histology. Rats of baclofen group showed mild features of hyperactivity using locomotor test according to the parameters of Moser *et al.* and of Molingo *et al.*^(24,25). During open field habituation test, the chronic administration with baclofen leads to hyperactivity as the total distance was greater in rats treated with baclofen compared to those of control rats. Our findings are in accordance with those of Stoppel *et al.*⁽¹³⁾. Moreover, the zone specific analysis revealed that rats treated with baclofen explored the outer zone more than the inner one when compared to those of control indicating features of an increased exploring.

Depending on the observations of the previous experiment which proposed that GABA-B receptors' activation could emerge the contrast in neuronal excitation in some zones of the brain⁽²⁹⁾. The anxiolytic influence of baclofen may reflect stimulation of GABA-B receptors of amygdala⁽³⁰⁾.

Object recognition test was done in this study and it revealed that rats of control group preferred the novel

object in comparison with those of baclofen group. However, non-significant differences among the index of discrimination of both groups. Moreover, the current study revealed that the rats of both groups spent more time for the familiar object and without a predilection of the new object. These observations are in accordance with those of other authors who reported that Vehicle-treated Wild type mice strongly preferred the novel object, and they observed that the prolonged intake of R-baclofen had no influence on predilection of novel object in these mice⁽¹³⁾. Their data suggested that R-baclofen may be useful for management of phenotypes of cognition in influenced human.

Histopathologic observations in this work research revealed some findings of brain tissues characterized by shrinkage of neurons with esinphelia and nuclear pyknosis in the cerebral cortex in rats of baclofen group, while controls showed normal brain histopathology. These observations were similar to those of others^(2,21). Penetration of the blood-brain barrier by baclofen lead to depressive neuronal activities in the central nervous system. In spite of the fact that baclofen's serum concentration is more than in brain, but its' elimination

from CNS is slower than that from serum. The depression of CNS , for that reason remains for several days, also beyond the returning of plasma baclofen levels to normal values which indicates marked neurotoxicity⁽³¹⁾.The raised lipophilic nature of baclofen is explaining the prolongation in coma after administration of its overdose⁽³²⁾.However, another study revealed that baclofen administration for 4 weeks did not induce any structural changes in the rats⁽²¹⁾.The difference between the findings of that work and our study may be the difference in the duration of exposure to baclofen.

In conclusions: Chronic administration of rats with baclofen (15mg/kg/day/2 months) induces changes in the brain histology , however, baclofen has no adverse

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effect on their behavior. According to our knowledge, this work is the 1st that tries to clarify the influence of baclofen on both the behavior and brains' histology. Our data suggesting that baclofen may have clinical utility as there is a need to make a good adjustment of the dose and careful monitoring especially when it was given for long duration. In addition, small doses of baclofen may be used in future to improve cognition.

Limitations of the study:

Several limitations were faced throughout this work. One of them is absence of well-equipped laboratory or place for testing of the behavior of rats in our College , and lack of advanced camera to recording their behavior especially at night.

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