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POTENTIAL ROLE OF NITRIC OXIDINEXPERIMENTAL-PILOCARPINE-INDUCEEPILEPSY IN ADULTMALEALBINO RATS
EPILEPSY ANDNITRICOXIDE

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Abstract

Epilepsy is the most common serious neurological condition affecting 1 to 2 % ofworldpopulation.Despite extensiveprogress,thereare still manyunansweredquestionsabout factors inducing epilepsy. Therefore, this work was carried out in an attempt to studythe effect of nitric oxide (NO) modulation on the development of epilepsy. Rats were dividedinto the following equal groups (8 rats each) according to treatment;Controlnontreated;Pilocarpinetreated;Pilocarpine+Diazepamtreated;Pilocarpine+Aminoguanidine(aselective inhibitor of inducible nitric oxide synthase; (iNOS) treated groups. Twenty-fourhours' rat observation and biochemical analysis of brain homogenates and serum showed thatPilocarpine induced seizures in all rats with high lethality associated with increased brainexcitatory transmitter; glutamate, Gamma aminobutyric acid (GABA), oxidative stress andNO,withincreasedseruminflammatorymediatorsastumornecrosisfactor(TNF-α).Complete protection was observed with Diazepam without reversal of Pilocarpine-inducedbrain excitatory transmitters changes apart from a significantly higher GABA levels but withreducedlevelsofbrainNO,MDA,andserumTNF-αlevels.Ontheotherhand,Aminoguanidine was protective and reversed all the Pilocarpine induced biochemical as wellas histological changes. In conclusion, although the role ofNOinthepathophysiology ofepilepsyiscontroversial,ourresultsshowedthatblockingiNOSwithAminoguanidineprovedtobeprotective,opening thewayforanewstrategyofantiepilepticmanagement.

Keywords: Epilepsy,Pilocarpine,Aminoguanidine,induciblenitricoxidesynthase(iNOS),tumornecrosisfactoralpha(TNF-α).

Introduction

Epilepsyisaneurologicaldisordercharacterizedbyrecurrentandunpredictableseizures. It is considered one of the most common chronic disorders affecting around 50million individuals of all ages worldwide. The incidence of epilepsy is higher in developingcountries compared to developed countries **(Sculier et al., 2018)**. Long term sequelae ofepilepsy may include neurological, cognitive, behavioral impairments, decline in quality oflife and exerts heavy burdens on the patient and the healthcare system. Outcomes depend ontype of epilepsy, type of status epilepticus (SE), etiology, SE duration, and patient's age**(OduahandIwanowski, 2020)**.Epileptogenesisisdefinedasaprocessthatleadstotheoccurrenceofthefirstspontaneous seizure and recurring epileptiform events afterthe brain insult.In addition,epileptogenesisincludesthedevelopmentandextensionoftissuecapableofgeneratingspontaneous seizures, including development of an epilepsy condition and progression afterthecondition isestablished**(Ambroginietal., 2019).**

Epileptogenesis frequently associate different brain insults characterized by increasedreactive oxygen and nitrogen species (ROS/RNS) generation and excitotoxicity mediated byincreasedglutamate.Oxidativestresstargetsmitochondrial DNAwithconsequentATPdepletion,produceslipidperoxidationofthenervecellmembranesthatfacilitatesdevelopment ofabnormal hyper-excitable circuits**(InderandVolpe,2018)**.In addition,disruption of blood brain barrier (BBB) and free movement of excitatory molecules betweenbloodandtheextracellularbrain fluidcould alsooccur**(Kadryetal.,2020)**.

Physiologicalconcentrationsofnitricoxide(NO)formedinthebrainregulatecerebral blood flow, as well as the activity of dopaminergic, glutaminergic, and GABAergicsystems, hormonal release, apoptosis, pain and analgesia, in addition to learning, memory andbehavioral circuits **(Reis et al., 2017)**. When formed in excess, by inducible NO synthase(iNOS)underpathologicalconditions,itbecomestoxicduetoformationoftoxicperoxynitrite radical with extensive nitrosylation of functional proteins. Due to this doublefacedcoinoffunctions,contradictoryresultshavebeenobtainedinvivo,inwhichmanipulation of the brain NO level was either pro-epileptic or antiepileptic **(Thiel, 2019)**.Therefore, this work was carried out in an attempt to

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study the effects of NO modulation onthedevelopmentofepilepsytryingtofindnewtherapeuticstrategies forsuchcondition.

Materialandmethods

I. **Animals:**

Thirty-two adult male albino rats, weighing (200- 250 gm), were obtained from theNational Research Center,Cairo; Egypt. Theywere housed instainlesssteel cagesthatoffered adequate space for free movement and wandering (40 cm x 40 cm x 25 cm) at roomtemperature with natural dark/light cycles, and allowed free access to water and commercialrat's diet (Nile Company, Egypt) for two weeks for acclimatization. Rats were fed a standarddiet of commercial rat chow and tap water ad libitum through the time of the study. Allexperimentswereperformedaccordingtoregulationsundertheappropriateanimallicenses approved by the animal care committee of Faculty of Medicine-Minia University, accordingtotheinternationalguidelines.

II. **Induction of Seizures:**

Allexperimentswereconductedinaquietlabwithconstantlightconditionbetween10 a.m. and 3 p.m.Following Pilocarpine injection, rats were put in individual cages, observedcloselyandcontinuouslyforonehourandfrequentlythereafterfor24hourswhentheexperimentwasterminated .Duringthisperiod,ratsweremonitoredforthefollowing:

a. The scoring was based on the Racine scale, as described previously **(Racine et al. 1972)** withthe following stages: stage (0); no abnormality, stage (1); Mouth and facial movements, stage(2); Head nodding, stage (3); Forelimb clonus, stage (4); Rearing,stage (5); Rearing andfalling.Afullmotorseizure,withtemporarylossofposturalcontrol,isreferredtoasaStage5motorseizure.
b. Percentage incidence of seizures (rats showing at least clonic spasms of the forelimbs wereconsideredpositive):[No.ofratsshowingseizure/No. ofrats pergroup] x100.
c. Timeofonsetofseizuresafter Pilocarpine;thelatentperiod.
d. Percentageofmortalityafterone and24hours.

III. **Experimentaldesign:**

Ratswere randomlyclassified into 4equal groups(8ratseach)asfollows:

1. **Controlgroup**;inwhichratswereleftfreelywanderingintheircageswithfreeaccesstofoodand water.
2. **Pilocarpinetreatedgroup**;inwhichratswereadministeredsingleintra-peritonealinjectionof Pilocarpine (400 mg/kg i.p.). Alpha methyl scopolamine was given subcutaneously in adose of 1 mg/kg as anti-muscarinic drug 30 minutes before pilocarpine to block its peripheralcholinergic side effects; salivation, dacryorrhea, diarrhea, bradycardia and hypotension. Itdoes not cross the BBB and does not interfere with the central effects of Pilocarpine onseizureinduction**(Curia etal. 2008)**.
3. **Pilocarpine + Diazepam treated group**; in which rats received a single injection of Diazepamas a reference anticonvulsant drug at a dose of 1 mg/kg, i.p., one hour before induction ofepilepsyby Pilocarpineasin group 2**(Zaeriand Emamghoreishi2015)**.
4. **Pilocarpine + Aminoguanidine treated group**; in which rats received a single injection ofAminoguanidine; a selective inhibitor of inducible NO synthase (iNOS) at a dose of 200mg/kg i.p. one hour before induction of epilepsy by Pilocarpine as in group 2 **(Byun et al.2009)**.

IV. **Drugprotocol:**

All chemicals used in the present study were purchased from Bio-diagnostic, Egypt, unlessmentionedotherwise.

V. **Biochemical analysis:**

- Blood samples were withdrawn from the retro-orbital venous plexus either immediately afterfirst seizure or at the end of the first hour for rats that didn't show seizures. Blood wasallowed to clot, centrifuged and sera were obtained and stored at -20 ºC for determination oftumornecrosis factor-α(TNF-α)byELISAmethod(PrechekBio,Inc.,India).
- At the end of the whole experimental period (24 hrs), the surviving rats were sacrificed bycervical dislocation. The heads of both sacrificed and dead rats were immediately dissectedandthebrains weregentlyremovedforpreparationofbrainhomogenates.
- Preparationofbrainhomogenatesforbiochemicalassay:Thebrainswerewashedwithnormal saline to remove blood and brain tissue samples from the hippocampus and temporallobe were weighed, homogenized in 10 volumes of cold phosphate buffered saline solution(PBS); pH 7.35, using ultrasonic homogenizer (4710 series, Chicago). The homogenate wasthencentrifugedincoolingcentrifugeat-4ºC,andthesupernatantwasusedfordetermination of: Malondialdehyde (MDA) as previously described **Ohkawa et al. (1979)**,Nitricoxide(NO)wasestimatedbyELISAkit(PrechekBio,Inc.,India.),Gamma-

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Aminobutyricacid(GABA)andglutamateusingELISAkit(BioAssaySystems,USA)accordingtothemanufacturer'sinstructions.

VI. **HistologicalExamination:**

Brain samples were fixed in 10 % formal saline for 24 hours, processed in ascendinggrades of ethanol, cleared in xylene, infiltrated, and embedded into blocks of paraffin. Serialsections (5- 6 µm thick) were cut and mounted on glass slides, stained with Hematoxylin &Eosin(H&E)tobeviewedbythelightmicroscope.

Statistical analysis

The analysis of the data was carried out using the IBM SPSS 20.0 statistical packagesoftware (IBM; Armonk, New York, USA). Analysis of variance (ANOVA) was used forcomparison between independent groups for parametric data followed by Tukey post hoc testtoassessintergroupdifferences.Ap-valueof0.05orless wasconsideredsignificant.

Results

1- Assessment of the effect of Pilocarpine with or without different treatments on the timeofonset, % incidenceof seizuresand % of deathsafter1 and 24 hours:

As shown in Table 1, single intraperitoneal injection of Pilocarpine produced tonic/clonicseizures in all experimental animals (n=8, % incidence 100%) after an average period of13.17±1.6 minutes with 75% deaths after 1 hour and 12.5% after 24 hours and only one ratsurvived till the end of experiment (24 hrs). Pilocarpine induced Seizures were completelypreventedwiththereferenceanticonvulsivedrug;Diazepampretreatment. NOmodulationshowedthatblockingiNOSwithAminoguanidineofferedprotection,manifested by a delay in seizure onset, a lowered number of deaths; within the first 24 hrs(onlyonerat) withhigher survivalnumber(7rats).

2- Assessment of the effect of Pilocarpine with or without different treatments onthedifferentserum andbrainparameters:

Theresultsofthe presentstudyasshowninTable 2demonstratedthat:

- Intra-peritoneal injection of Pilocarpine produced a significant rise of serum TNF-α level ascompared with control group. Pretreatments with Diazepam and Aminoguanidine, 1hr beforePilocarpine, produced no significant changes in serum TNF-α level as compared with controlgroup.
- Pilocarpine treatment produced higher brain MDA level as compared to the control levels.Pretreatment with Diazepam and Aminoguanidine, 1hr before pilocarpine, attenuatedthePilocarpine-induced riseinbrainMDAlevels.Theselevelswereinsignificantfromthecorrespondingcontrollevels.
- Pilocarpine treatment produced significant higher glutamate level in the brain as compared tothe control group. Diazepam pretreatment, 1hr before Pilocarpine, produced non-significantdifference in brain glutamate than that of the only Pilocarpine injected group. On the otherhand, blocking NO synthesis with Aminoguanidine pretreatment, 1hr beforePilocarpine,counteractedtheeffectsofPilocarpineandproducedbrainglutamatelevelsthatwereinsignificantfromthecontrolgroup.
- Pilocarpine treatment produced significant higher GABA levels in the brain as compared tothe control group. Diazepam pretreatment, 1hr before Pilocarpine, significantly producedhigher GABA level than that of the only pilocarpine injected group. On the other hand,blockingNOsynthesiswithAminoguanidinepretreatmentcounteractedtheeffectsofPilocarpine and produced higher brain GABA levels that were significant from the controlgroup.
- Pilocarpine produced a significantly higher brain NO level than the control group; an effectthat was partially but significantly reversed by Diazepam. On the other hand, the selectiveiNOS inhibitor; Aminoguanidine completely blocked the effects of Pilocarpine and decreasedbrainNOlevelstothecontrollevel.

3- Histologicalassessment:

- Hippocampus formation appeared formed of the Cornu Ammonis (CA) and the dentate gyrus(DG). The cellular organization in zone CA1 was arranged as 3–4 layers composed of closelypackedsmallpyramidalneuronswithvesicularnuclei.Lighteosinophilicneuropilbackgroundisseen,containing neuronalandglialcellprocessesandsparseneuroglialcells.In contrast, in CA3 cells are loosely packed large pyramidal neurons with vesicular nuclei.The principal neurons of the dentate gyrus form a C-shaped band of densely packed columnsof granular cells, with vesicular nuclei and only few neuroglial cells were observed in theneuropil(Figure1 A-C).
- The result of the present studyshowed that the hippocampusappeared sclerotic showingmore extensive neuronal lossand some distinctive histo-pathological changesin the rats ofthepilocarpinegroup(Figure1 D-E).
- In Diazepam treated group, the hippocampal showed more or less normal morphologicalcharactersifcomparedtothatofPilocarpine-treatedgroup (Figure1F-H).
- In Aminoguanidine group, improved histological appearance of pyramidal and granular cellsthat

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appearedintactwithsporadic fociofdegeneratedandpyknoticcells(Figure1I-K).

Discussion

Epilepsy isoneof themostcommon neurologicconditions,with anincidenceofapproximately50newcasesper100,000populationsperyear.Approximately75%ofepilepsy begins during childhood, reflecting the heightened susceptibility of the developingbrain to seizures. So that, epilepsy is one of the most common and disabling neurologicconditions and a better understanding of epileptogenesis is the only way for development ofnew antiepileptictreatments**(Ułamek-Koziołetal., 2019)**.

The Pilocarpine-induced epilepsy rat model is the most appropriate and experimentalmodel simulating temporal lobe epilepsy in humans; so, it is commonly used to study itspatho-physiologic mechanisms and the potency of antiepileptic drugs (AEDs) **(Devinsky etal.,2018)**. Thatiswhy weused this modelinthepresentstudy.

Inthepresentstudy,intraperitonealinjectionofPilocarpineproducedtonic/clonicseizures in all experimental animals with high mortality rate. In addition, Pilocarpine inducedhistopathological changes in the hippocampus. These data is compatible with other studiesreportedthatPilocarpineinducesepilepsybyactingonbrainM_1muscarinicreceptors,especially in the hippocampal region and triggers an imbalance of excitatory and inhibitorytransmitterreleasein favorof theformer**(Maia etal., 2020)**.

In the present work, Diazepam pretreatment completely prevented the occurrence ofpilocarpine-induced seizure. However, the inhibitory GABA levels were significantly higher.So, diazepamcould actthroughpotentiating GABAsynthesis,release orattenuatingitsbreakdowntobalanceglutamateexcitotoxicity**(Lorenz-Guertin,2019)**.

Additionally, in the present study, the selective iNOS inhibitor; Aminoguanidine wasfound to be antiepileptic. The incidence was reduced and a significantly longer delay of onsetwas observed with minimal lethality. The biochemical changes induced by Pilocarpine werecompletely reversed with no statistical differences with the control. As far as we know, noprevious studies have documented a convulsant effect for NO donors or for NOS modulators,so,NOcouldnotbeaninducerofepilepsyasPilocarpine.However,itcouldplayapermissive role with other inducers. It increases glutamate release by activating presynapticCa^{+2} channels **(Caviedes et al., 2020)**. On the other hand, inhibition of glutamine synthase bys-nitrosylation prevents inactivation of glutamate and increases excitotoxicity **(Nagel andEisel,2021)**.ThisexplainswhyselectiveblockingofiNOSwithAminoguanidineinthisworkwasfound to beprotective.

Inthepresentstudy,Pilocarpine-inducedseizureinducedoxidativestressthatismanifested by the significant high brain MDA levels than the control levels. In addition,inthe present study, iNOS inhibition by Aminoguanidine and Diazepam were associated withinsignificant high MDA level. Reactive oxygen species are generated during epilepsy, as inthe present data, through the following mechanisms: a) increased mitochondrial oxidativephosphorylation,andb)increasedbraincatecholaminetransmission,speciallydopamineduring seizure and their catabolism by monoamine oxidase (MAO) is another mechanism forgenerating ROS during epilepsy **(Shishmanova-Doseva et al., 2021)**. These mechanisms mayexplainthedataof thecurrentstudy.

The data of the present work showed significantly higher serum TNF-α level with thePilocarpine group than control group that is compatible with previous studies **(Han et al.,2018)**. Pretreatments with Diazepam and Aminoguanidine, 1hr before Pilocarpine, producednosignificantchangesinserumTNF-αlevelascomparedwithcontrolgroup.TNF-αenhancestheexpressionofendothelialadhesionmoleculesandincreasescapillarypermeability,resultingintheinfiltration ofinflammatorycellstotheaffectedsiteandeventual tissue necrosis. Elevated TNF-α level also decreases inhibitory transmission **(Mengand Yao, 2020)**.

In the current work, histological examination of brains resected from rats followingseizuresattheendoftheexperimentrevealedthat;inPilocarpinetreatedgroup,thehippocampus appeared sclerotic showing more extensive neuronal loss and some distinctivehisto-pathological changes. Similar results were obtained in epileptic rodents, either aftertraumaticbraininjuryoraftersystemicPilocarpineinjection**(Paulettietal.,2017)**.Inaddition,thepresentstudyshowsthatDiazepam1hrbeforePilocarpine,differenthippocampalareasshowedimprovementinthedegenerativechanges observedinPilocarpine-treated group. **Juvale and Che Has (2021)** reported that Diazepam can protectagainstPilocarpineinducedepilepsythroughdecreasingneuronalexcitation,inhibitinginflammatoryreactionsandastrocytesactivation.

Additionally, the present study showed that iNOS inhibition through Aminoguanidinecan protect the hippocampus against Pilocarpine induced epilepsy. This is compatible withother study reported that decreased NO level, through NOS inhibitors, reduced the severity ofstatus epilepticus and hippocampal changes in lithium–Pilocarpine models**(Eslami et al.,2021)**.

In conclusion, according to the data of the present study, the major mechanisms ofseizuresandepilepsydevelopmentmayincludeincreasingneuronexcitabilityandinflammation as reported with increased brain excitatory chemical transmitters, MDA, withserum TNF-α, while these levels were decreased

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with the protective drug; Diazepam. Inaddition,Aminoguanidine,theiNOSinhibitor,provedtobeanti-epilepticsoNOisproepileptic.Thus,inhibitorsofiNOScouldbeanew antiepilepticstrategy.

Recommendations

The results ofthe present study could openthe wayformore researchesto studytheuse of different iNOS inhibitors in treatment plans of epilepsy alone or combined with otheranti-epilepticdrugs.

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Declarationofinterest:

The authors declare that there is no conflict of interest that could be perceived as prejudicingtheimpartialityoftheresearchreported.

Ethicalapproval:

All applicable international, national, and/or institutional guidelines for the care and use ofanimals were followed according to the guidelines of the Animal Care and Use Committee ofFacultyofMedicine,MiniaUniversity,Minia,Egypt.

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Table1: Effectofpilocarpinewithandwithoutdifferenttreatments ontheonset,%incidenceof seizuresand % ofdeathsafter1 and 24hours:

Groups / Parameters	Control	Piloc.	Diaz. + Piloc.	Aminog. + Piloc.
Onsetofseizures(min)	Nosei zure	13.17 ±1.6	Nosei zure	33.04* ±3.09
%Incidenceofseizures	0rats 0 %	8rats 100%	0rats 0 %	3rats 37.5%
No.&%ofdeathsduring1st hr.	0rats 0 %	6rats 75%	0rats 0 %	0rats 0 %
No.&%ofdeaths between1and24hr	0rats 0 %	1rat 12.5%	0rats 0 %	1rat 12.5%
No.&% ofrats survivingtotheend	8rats 100%	1rat 12.5%	8rats 100%	7rats 87.5%

Dataareexpressedasmean±SEMof8ratsineachgroup.Piloc:Pilocarpine;Diaz:Diazepam;Aminog:Aminoguanidine.*Significantatpvalue≤0.05.ANOVAfollowedbyTukeypost-hoctest.

Table 2: Effect of pilocarpine with and without different treatments on different serum andbrainparameters:

Groups / Parameters	Control	Piloc.	Diaz. + Piloc.	Aminog. + Piloc.
SerumTNF-αlevel(Pg/ml)	1.252±0.01	2.011±0.173*	1.320±0.034*	1.264±0.043*
Brain MDA level(nmol/gmtissue)	11.59±0.26	16.97±0.19*	12.73±0.105*	9.93±0.05*
Brainglutamatelevel(mg/ gm tissue)	4.095±0.04	9.94±0.15*	9.86±0.09*	3.84±0.06
BrainGABAlevel(Pg/ gm tissue)	21.11±0.295	62.22±0.435*	118.55±0.495*	29.085±0.295*

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Brain NO level(µmol/gmtissue)	19.71±0.125	32.94±0.125*	25.16±0.255*	19.74±0.125

Dataareexpressedasmean±SEMof8ratsineachgroup.Piloc:Pilocarpine;Diaz:Diazepam;Aminog:Aminoguanidine.*Significantatpvalue≤0.05.ANOVAfollowed byTukeypost-hoctest.

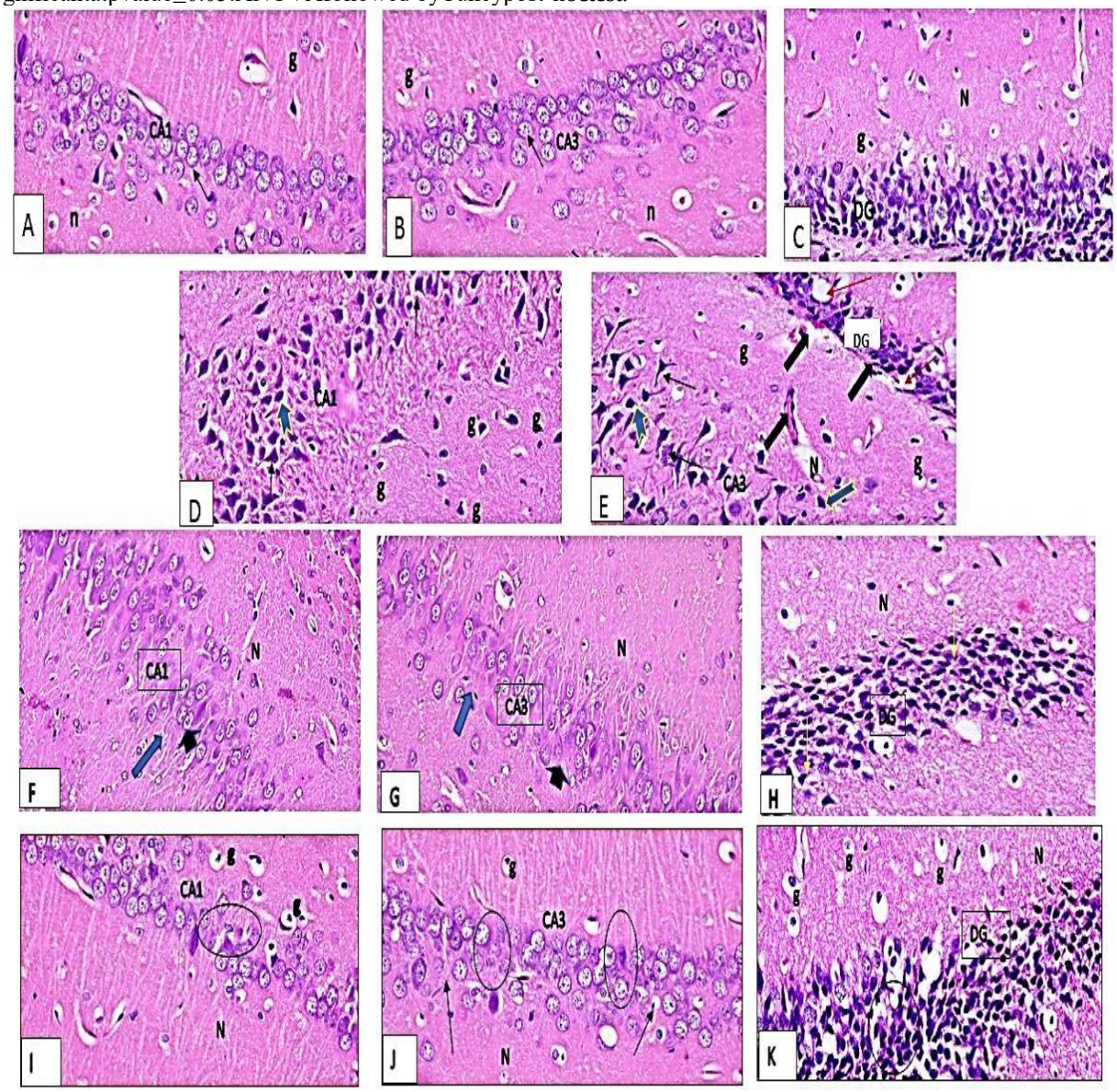

Figure 1. A-C) Control group: CA1 (A) and CA3 (B) showing small closely and large loosely packedpyramidalneuronsrespectively(arrows).DG(C)showingcolumnsofgranularcells.Noticeneuropil (n) and sparse glial cells (g).**D-E) Pilocarpine-treated group:**CA1 (D) and CA3 (E) showingshrunken pyramidal cells (thin arrows). DG (F) showing vacuolated (red arrows) and deeply stainedgranular cells (tailed arrows). Congested blood vessel with extravasated RBCs (thick arrows) andvacuolated neuropil (N).**F- H) Diazepam treated group:** CA1 (J) and CA3 (K) showing pyramidalneurons(thickarrows),fewdeeplystainedcells(arrowhead)andnormalneuropil(n).DG(L)showingnormalgranularcells(thickarrows).**I-K)Aminoguanidine-treatedgroup:**normalpyramidal and granular cells (arrows), sporadic foci of degenerated and pyknotic cells (empty circles).Neuropil(N)showsproliferationofneurogliacells(g).