RESEARCH ARTICLE

Protection Anticonvulsant effect of Irbesartan in Lidocaine Induced Seizure in Mice

Huda N. Tehewel[1], Shihab A. Shihab[2]

[1]College of Pharmacy, Al-Yarmouk University College, Baghdad, Iraq
[2]Department of Pharmacology and therapeutics, Al-Nahrain University, Baghdad, Iraq

Received: 26th Dec, 2019; Revised: 23rd Jan, 2020; Accepted: 22nd Feb, 2020; Available Online: 25th Mar, 2020

ABSTRACT

Background: Epilepsy is one of the most common serious brain disorders and may have many possible causes due to many different cellulars or biochemical changes such as alterations in ion channels function, neurotransmitter level, neurotransmitter receptor function, energy metabolism, and oxidative stress. The chronic and intermittent nature of epilepsy and the inability of the current drugs to sustain long term remission made a continuous need to investigate a new drug that aims to minimize the morbidity and to improve the quality of patient life.

Aim of the study: To investigate protection anticonvulsant effects of irbesartan in lidocaine induced Seizures in male mice.

Methods: A total of 40 albino male mice were equally divided into four groups. The blank group was given vehicles only. The model of seizure was induced by a single intraperitoneal injection of Lidocaine (75 mg/kg). Irbesartan group was pretreated intraperitoneally (20 mg/kg) for 7 days and lidocaine injection on the 8th day as an acute induction, and the same was applied to the diazepam group (2 mg/kg) which served as a positive control. On the 8th day, the level of N-methyl-D-aspartate receptor (NMDA-R) and caspase-3 were measured. Furthermore, the body weights were daily documented before each administration.

Results: The results revealed a highly comparable significant reduction in NMDA-R and Caspase-3 in both irbesartan and diazepam groups, also both modestly decreased in potency and duration of the seizure.

Conclusion: Irbesartan significantly reduces NMDA-receptor and Caspase-3 in lidocaine induced seizure in mice model and decrease the potency and duration of the seizure.

Keywords: N-methyl-D-aspartate.

International Journal of Pharmaceutical Quality Assurance (2020); DOI: 10.25258/ijpqa.11.1.15

How to cite this article: Tehewel HN, Shihab SA. Protection Anticonvulsant effect of Irbesartan in Lidocaine Induced Seizure in Mice. International Journal of Pharmaceutical Quality Assurance. 2020;10(1):101-108.

Source of support: Nil

Conflict of interest: None

INTRODUCTION

Epilepsy is a heterogeneous sign characterized by recurrent seizures; seizures are limited events of brain dysfunction resulting from an infrequent discharge of cerebral neurons, it affects about 1% of the world's population common neurologic disease after headache and stroke, the causes of seizures are many, involve the full scope of neurologic diseases from infection to neoplasm and head injury, in some subgroups, heredity has demonstrated to be a predominant factor, single gene defects, involving genes coding voltage-gated ion channels or gamma-aminobutyric acid (GABA) receptors.[1]

The utilization of antiepileptic drugs is restricted because of the immense range of inconvenient impacts. For example, subjective inability, compelling scatters, and recurrent seizures.[2] Thus, there is a requirement for the improvement of new antiepileptic drugs with less antagonistic impacts and high adequacy.

Irbesartan is an angiotensin receptor blocker (ARB) used largely for the management of hypertension; it competes with angiotensin II for binding at the AT1 receptor subtype.[3]

Recently, recognized that ARBs are neuroprotective neuroprotection may be the result of direct blockade of brain AT1Rs, a consequence of AT1R blockade outside of the brain, or respond to processes additional to the class effect of AT1R antagonism. The ARBs improve inflammatory and apoptotic responses to glutamate, interleukin 1β, and bacterial endotoxin in cultured neurons, astrocytes, microglia, and endothelial cerebrovascular cells.[4]

The anti-inflammatory, anti-apoptotic effect, and NMDA-receptor blocker of irbesartan suggest new promising therapeutic perspective against several brain disorder

*Author for Correspondence: nadanahi9@gmail.com

MATERIALS AND METHODS

Materials

Irbesartan powder (Sigma-Aldrich, USA), Carboxymethyl cellulose sodium salt (CMC-Na) (Panreac, Spain), lidocaine vial (Ajanta, India), caspase anti-body kit (Abcam, UK), GRIN2B (phosphor-Tyr1336) antibody kit (Abcam, UK), diazepam ampule (Roche, Germany). All other chemicals are of the highest purity obtained from reputed sources. Animal feeding needle curved 20G×1″ (2 mm tip diameter) Reusable (Petsurgical, India).

Preparation of Irbesartan Stock Solution

Irbesartan powder purchased from (Sigma-Aldrich), pure Irbesartan powder weighted by using the electronic balance in a beaker and dissolved in CMC-Na 0.5% w/v colloidal solution according to the United States Pharmacopeia to get final concentration of Irbesartan; allowing a dose of 0.3 ml/30 g body weight.[5]

The protection anticonvulsant effect against seizure model was done by a single daily intraperitoneal (i.p.) injection of irbesartan, at a dose of 20mg/kg[14] in 0.5% w/v CMC-Na/normal saline, for 7 day before seizure induction.[6]

Lidocaine-induce Status Epilepticus

Lidocaine assists the incidence of status epilepticus due to lidocaine increases the intracellular concentration of calcium in the brain that may illuminate its potential neurotoxicity.[7] Status epilepticus was characterized as a period of nonstop seizures that went on for no less than 5 minutes or seizures that repeated at short interims (<1 min) setting up a continuing epileptiform condition.[8] In a highly concentration dose, depressed GABA by binding to the GABA discrimination site and to an additional site in the GABA-ionophore receptor complex.[9] Also, Lidocaine-induced convulsive seizures are intermediate via excitatory glutamate transmission through together NMDA and non-NMDA receptor systems,[10] and may causing oxidative stress in the hippocampus and amygdala of adult rats through it is effects on the antioxidant enzymatic system and causes a reduced concentration of GSH while the lipid peroxidation increased.[11]

Animals

A total of 40 apparently healthy albino male mice weighing between 25 and 30 g were included in the study and were randomly divided into four groups (10 mice each). The mice were obtained from the National Center for Drug Control (NCDC) and Research/Ministry of Health/Baghdad. Each group was kept in a separate plastic cage, had free access to water and pellet diet maintained at 12 h light/dark cycles with room temperature (22–25°C), and habituated for a week before starting with the *in vivo* study.

The Institutional Review Board approved the study protocol at the College of Medicine, Al-Nahrain University. The study was conducted from December 2018 through June 2019 at the department of pharmacology/animal house- college of Pharmacy, Baghdad University and pathology–College of Medicine, AL Nahrain University.

Pretreatment Tests

Four groups of mice were utilized for the pretreatment tests:

Each group contained 10 mice were taken standard (Diazepam) and Irbesartan for 7 days intraperitoneally, before lidocaine (75mg/kg) induction

- *Group 1:* (Normal group): This group did not get any medication was filled in as a typical control group to distinguish the normal values.
- *Group 2:* (lidocaine induced epilepsy group only) were taken lidocaine injection intraperitoneally (75 mg/kg)[(12)], considered as a negative control group.
- *Group 3:* They were taken (2mg/kg)[(13)] of diazepam (I.P.) 7 days before lidocaine injection.
- *Group 4:* They were injected (20mg/kg), of Irbesartan (I.P) 7days before lidocaine injection.

Tissue Processing

The brain of each mouse was quickly and carefully surgically removed on the 8th day of study after induction with lidocaine (except normal control group), after killing and fixed in 10 % formalin for Immunohistochemistry studying.

Parameters of Study

1. Behavioral changes assessment.
2. After lidocaine injection, each mice was carefully estimated by noticing the onset of the first seizure, duration, recurrent seizures, and death, listed by naked eyes.
3. Caspase-3 level in hippocampus neurons.
4. Oxidative stress assessment through NMDA-Receptor.

Immunohistochemistry

Principles of the test Immunohistochemistry (IHC)

This procedure based on the antigen detection using a specific antibody, which binds to specific targeted protein, the bound primary antibody identified by secondary antibody conjugated with biotin, secondary antibody is then distinguished by streptavidin conjugated with horseradish peroxidase polymer that catalyzes the substrate H2O2 into free oxygen and water (H_2O), then, the free oxygen oxidize 3, 3`-diaminobenzidine (DAB) into dark brown precipitates, a positive reaction will have showed as a brown-colored precipitate at the antigen site counterstained with hematoxylin then tested for immunoreactivity[15]

Immunohistochemical (IHC) Procedure

A. Serial thin sections of (5µm) thickness were done for each paraffin-embedded tissue block; the sticking of each section on charged slides was done. Sections were left to dry to encourage grip between the section and the charged glass surface.

B. Deparaffinization and rehydration: this progression include:
   1. Dewaxing of the paraffin inserted section was put inside a hot air broiler at 65°C for 30 minutes.
   2. Deparaffinization was finished by inundating the slides in xylene for 5 minutes then in new xylene for 5 minutes.

3. Rehydration of tissue area achieved through drenching of slides in successive weakening's of ethanol as the accompanying request:
   I. Absolute ethanol for 5 minutes.
   II. 95% of ethanol for 5 minutes.
   III. 90% of ethanol for 5 minutes.
   IV. 70% of ethanol for 5 minutes.
   V. 50% of ethanol for 5 minutes.
   VI. Distilled water for 5 minutes.

C. Define a suitable area (by pap pen) from the slide which was tested by IHC. Enough drops of hydrogen peroxide block were added to cover the section (incubated at 25°C for 10 minutes). At that point, the slides were flushed with refined water, depleted, and smudged delicately. Protein block was applied and incubated at 25°C for 10 minutes to block nonspecific background staining. Slides then washed in Tris buffer saline (TBS) for 5 minutes. Primary AB was added to the slides and incubated overnight in the humidified chamber. Then slides were washed with phosphate buffer 3 times, and secondary AB (complement) was added and incubated at 25°C for 10 minutes, washing was done with phosphate buffer (2 times).

D. HRP was applied to the tissue and slides were incubated at 25°C/15 minutes. Then washed with phosphate buffer saline. Tissue sections were treated with diluted liquid DAB for 1-10 minutes at room temperature (70 µL of 30 µL (1 drop) DAP chromogen to 1.5 ml (50 drops) of DAP substrate, mix by swirling and apply to tissue. Slides were washed with phosphate buffer saline (4 times).

E. Counterstaining was done with Myers´ Hematoxylin for 2min, washing with tap water was then followed. Then Section was mounted` with super mount and coverslip added and examined under a light microscope.[16]

Table 1: Mean IHC score of oxidative stress, anti-apoptotic, and anti-inflammatory markers in induced and control group.

Variables (score)	*Lidocaine*	*Controls*	*p-value*
Caspase-3	3.13 ± 0.64	0.25 ± 0.16	<0.001
NMDA	2.75 ± 0.46	0.25 ± 0.16	<0.001

NMD: N-methyl D-aspartate.

Table 2: Mean IHC score of oxidative stress, anti-apoptotic and anti-inflammatory markers in Irbesartan-treated group compared with induced and control group

Variables (score)	*Irbesartan*	*Lidocaine*	*Controls*	*p-value*
Caspase-3	0.88 ± 0.64	3.13 ± 0.64	0.25 ± 0.16	<0.001
NMDA	0.25 ± 0.16	2.75 ± 0.46	0.25 ± 0.16	<0.001

NMD: N-methyl D-aspartate.

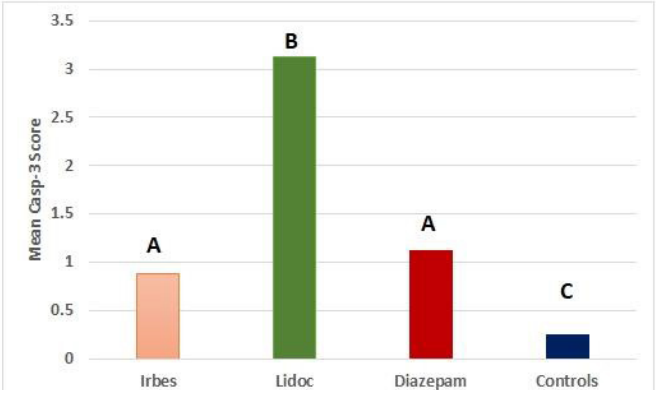

Figure 1: Mean caspase-3 IHC scores in different groups.

Evaluation of the Immunostaining

Evaluation of IHC results for performed by light microscope (Genex 20, America) at 40X objective lens with a total power of magnification 400X. Slides examined with a microscope to determine the level of neurodegeneration in the hippocampus.

Statistical analysis

Statistical Package for Social Sciences (SPSS version 25.0) was used for data analysis, and Microsoft Excel to generate graphs. Variables were expressed as a mean ± standard deviation (SD). When there was 2-group comparison, student test (t-test) was used to compare means, while analysis of variance (ANOVA) was used to compare means when there was a more than 2-group comparison. Spearman's rank-order correlation, as well as linear regression, were used to explore the correlations between different variables. The statistical tests were 2-sided, and a $p \leq 0.05$ was considered statistically significant.

RESULTS

Effect of Lidocaine on IHC Score of Oxidative Stress, Anti-Apoptotic and Anti-Inflammatory Markers

Table 3: Mean IHC score of oxidative stress, anti-apoptotic and anti-inflammatory markers in an irbesartan-treated group compared with diazepam-treated group

Variables (score)	*Lidocaine*	*Controls*	*p-value*
Caspase-3	0.88 ± 0.64	1.13 ± 0.35	<0.001
NMDA	0.25 ± 0.16	0.63 ± 0.42	<0.001

NMD: N-methyl D-aspartate.

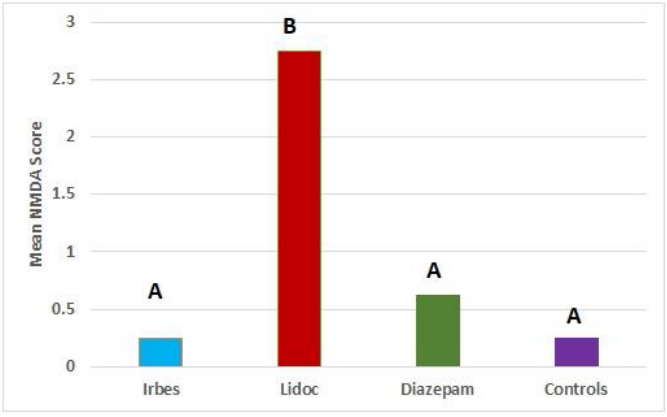

Figure 2: Mean NMDA IHC scores in different groups.

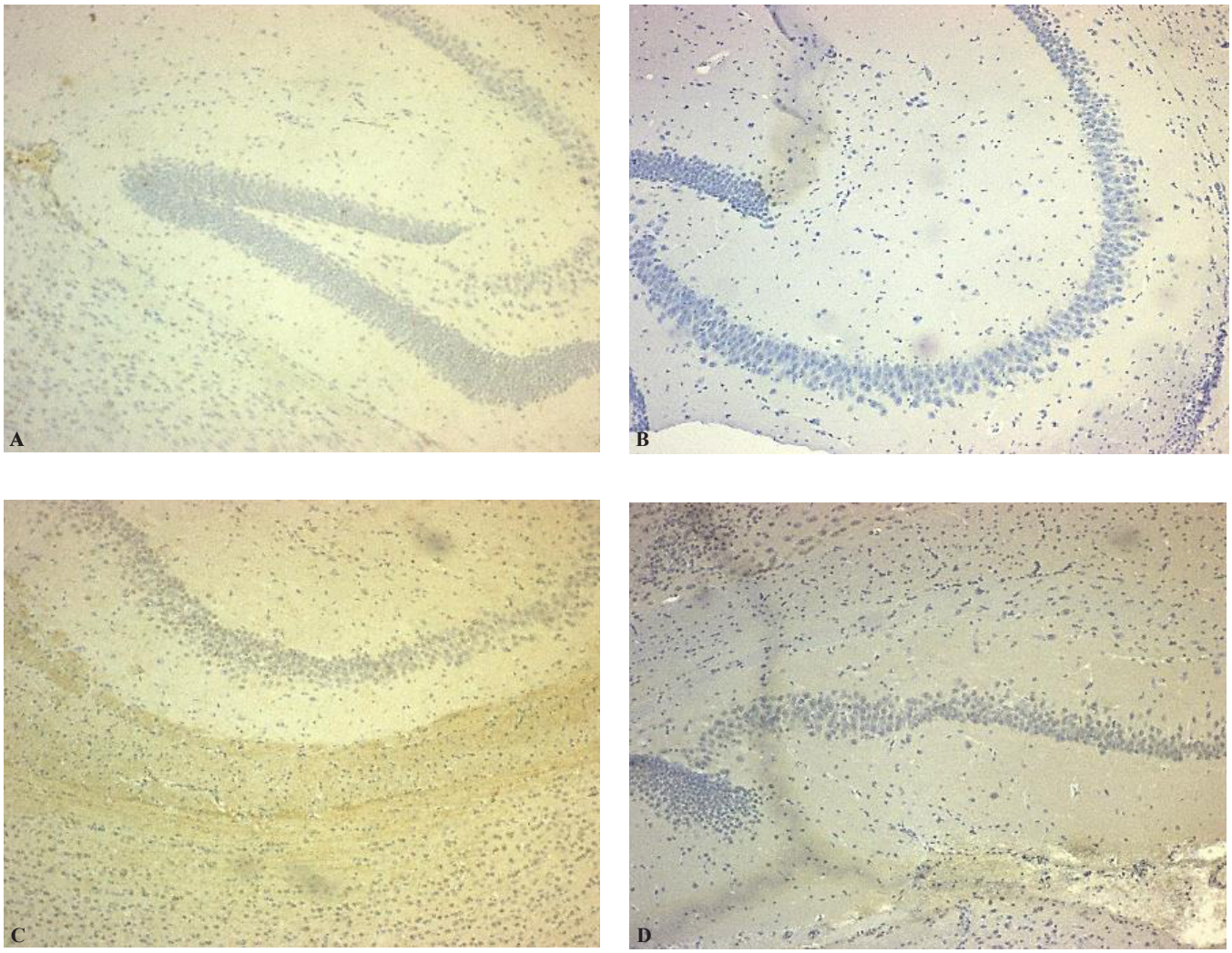

Figure 3 A to D: Immunohistochemical expression of Caspase-3 A) Normal tissue showed no intensity of caspase-3 (10X); (B) Induce tissue shown high intensity of caspase-3 (10X); (C) Irbesartan treated tissue shown low intensity of caspase-3 (10X); (D) Diazepam treated tissue shown mild intensity of caspase-3 (10X)

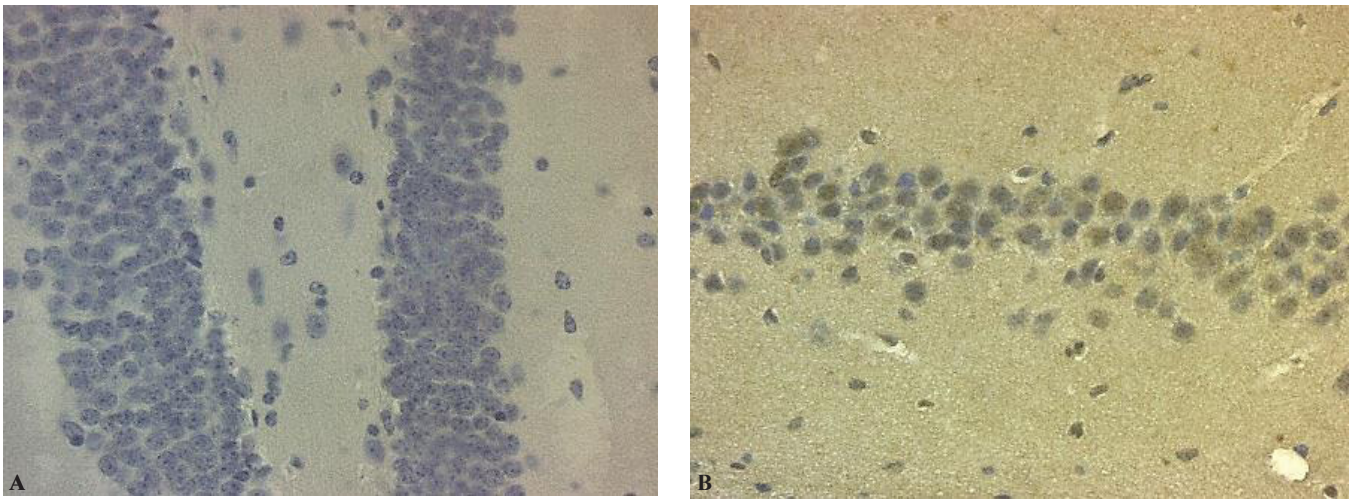

Figure 4 A and B: Immunohistochemical expression of NMDA; (A) Normal tissue showed no intensity of NMDA (40X); (B) Induce tissue shown high intensity of NMDA (40X)

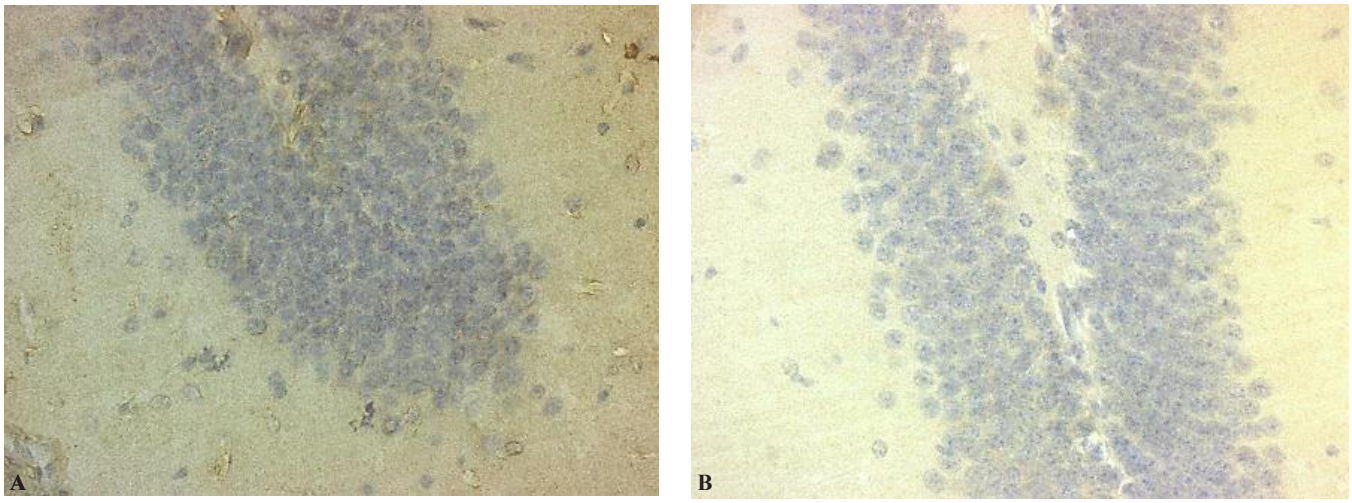

Figure 4C and D: Immunohistochemical expression of NMDA; (C) Irbesartan treated tissue shown low intensity of NMDA (40X); (D) Diazepam treated tissue shown mild intensity of NMDA (40X)

In this study, lidocaine (75 mg/kg)- was used to induce epilepsy. A comparison of a lidocaine-treated group with a control group revealed a highly significant increase in IHC score of each Capase-3 (3.13±0.64), and NMDA (2.75±0.46). (Table 3-1, Figures 1, 2)

Effect of Irbesartan on IHC Score of Oxidative Stress, Anti-Apoptotic and Anti-Inflammatory Markers

Mean NMDA IHC score in the irbesartan-treated group was (0.25 ± 0.16), was identical for that in control groups, and much lower than that of the induced group with a highly significant difference. Caspase-3 showed significantly higher IHC score (0.88 ± 0.64) than control but much lower than the induced group with a highly significant difference. (Table 2, Figure 1, 2).

Irbesartan Versus Diazepam

The effect of irbesartan on the IHC Score of oxidative stress, anti-apoptotic, and anti-inflammatory markers was compared with diazepam as one of the most commonly used anticonvulsant drug. Table 3-5 shows a comparison in this effect between irbesartan and diazepam. Each of caspase-3 and NMDA had slightly less IHC score in an irbesartan-treated group than that recorded in a diazepam-treated group. Mean IHC of NMDA in irbesartan- and diazepam-treated was 0.25 ± 0.16 and 0.63 ± 0.42 with no significant difference. (Table 3, Figure 1, 2).

DISCUSSION

Epilepsy is the most common neurological disease differentiate by several symptoms, including alterations of behaviors and consciousness, continued alteration in brain function.[17]
Lidocaine Consider a useful in animal model for studying mechanisms and therapeutic methods to epilepsy, in this model, excessive and persistent stimulation of cholinergic receptors lead to seizure-related brain damage in mice.[18]

The first step for seizure activity is the stimulation of muscarinic receptors, while, glutamatergic, GABA ergic, dopamine, adenosine, and serotonin appear to mediate seizure propagation and maintenance in rodent epilepsy models.[19]

Central excitation is produced by local anesthetics through suppressing inhibitory neurotransmission, the activation of excitatory amino acid systems indicated to be the basis for seizures induced by local anesthetics such as lidocaine.[20]

A seizure is a most commonly encountered toxicity that results from a high dose of lidocaine, the toxicological basis of anesthetic induce convulsion is referred to the ability of this agent to suppress inhibitory neuronal function that results from a decrease in presynaptic GABA release, which results in an exaggeration of excitatory neuronal function.[21]

This present study, interested with Immunohistochemical change of the following markers, MDA and caspase3 in the brain associated with lidocaine induce seizure (75 mg/kg I.P.) in mouse model we found a significant increase in expression of these markers in this group in comparison with a control group. Also, it caused generalized limbic seizures after approximately (4) minutes.

Effect of lidocaine versus a control group

Caspase-3 Effect

Several apoptosis pathways were identified in mammals that involve members of caspase gene families; one of them is caspase-3, which is a cysteine protease, that cleaves specific aspartate residues in proteins in an assortment of structural, housekeeping and regulatory proteins, these proteolytic actions lead to death.[22] Caspase-3 produced as a latent proenzyme, and proteolytically transfer to the active form in response to a numeral of apoptotic stimuli; caspase-3 protease activity is supposed to be a contributing factor in a several of neurodegenerative diseases.[22]

Since activation of caspase-3-like cysteine proteases shows a crucial role in neuronal apoptosis,[23] We explored the possibility that SE induced by systemic lidocaine through immunohistochemical data, by examining the distribution

of the activated form of the protein in the hippocampus of mice brain, which indicate the overexpression of caspase-3 in degenerative cell after lidocaine induction, is involved in the neuronal apoptotic and cell death.

In our study, there was higher expression of caspase-3 in induction group (Lidocaine-administrated group) in comparison to control group (Distill water-administrated group), in which p-value was <0.001 (As in Table 1 Figure 1, 3), which indicate significant differences between the expression of the protein between the two groups, and this may be due to the effect of lidocaine administration, which is responsible for the increase of expression of caspase 3, that is involved in the neuronal apoptotic and cell death, occurs in the hippocampus regions through which the seizure propagates, and this mean caspase-3 like proteases is an important agent in the process of neuronal apoptosis and regulate neuronal programmed cell death after lidocaine induction, and this happen through the combination of a change in signaling molecules and structural changes.[23]

NMDA Effect

Variations in inhibitory and excitatory neurotransmission show a central role in the etiology of epilepsy, in which, overstimulation of glutamate receptors influencing epileptic activity and neuronal damage, N-methyl-D-aspartate (NMDA) receptors, is one of the class of ionotropic glutamate receptors, has a primary role in this process.[24]

Lidocaine make central excitation via suppressing inhibitory neurotransmission.[25]

Here we found an increase in NMDA receptor expression in lidocaine group in comparison to control group, in which p-value was <0.001 (As in Table 1, Figure 2, 4), and this may result in overexcitation of glutamate transmission which mediated by N-methyl-D-aspartate receptor and suppressing inhibitory neurotransmission that have been shown to have strong effects on the of the brain.[26,27]

Effect of Irbesartan

Neuroprotection of irbesartan may be due to the direct blockade of brain AT1Rs, a result of AT1R blockade external of the brain, or respond to procedures additional to the class effect of AT1R blockade.[28] The strongest evidence for direct diffusion into the brain after administration systemically, reaching therapeutically related to concentrations, has been described for ATRs in humans and rats.[29] Indirect effects in parenchymal neurons and glia are detected after systemic administration and involved auto-radiographic evidence of central AT1R blockade, Ang II effects blockade that administered directly into the brain, and decrease of brain parenchymal inflammation.[30]

Caspase-3 Effect

In this research, we observed a decrease in expression of caspase-3 in irbesartan treated group in comparison with lidocaine induction group, a difference that was more significant between two groups, in which p-value was <0.001 (Table 2, Figure 1,3), and this effect may be due to irbesartan administration, that act as ATRs blocker, decrease apoptosis through protects survival pathway PI3K/Akt/GSK-3β which is a cell survival pathway that suppresses caspase-3 and prevents apoptosis.[31] Akt is a serine/threonine protein kinase. PI3K can improve the activity of p-Akt mediated by phosphoinositide-dependent kinase1, which affects the phosphorylation of the downstream protein GSK3β. Apoptosis induces by activation of the Akt/GSK3β pathway by phosphorylation of the anti-apoptotic protein,[32] the higher expression of active GSK3β has been showing to stimulate neuronal apoptosis.[33] Previous research has suggested that the inhibition of GSK3β supports cell survival, irbesartan administration inhibited neuronal damage may be due to by inhibiting the activation of caspase-3, and inhibit the activation Akt/GSK3β pathway, and therefore may minimize apoptosis in the brain[(33)] and enhance PPARγ activation.[34]

NMDA effect

Our results demonstrated the expression of NMDA receptor in irbesartan treated group was identical to a normal group and much lower in comparison to lidocaine induction group by using IHC, in which p-value was <0.001 (Table 2, Figure 2,4), This highly significant difference may be due to the effect of irbesartan, through which reduces neuronal injury produced via NMDA receptor stimulation through neurotoxic glutamate concentrations,[35] neuroprotection mechanisms include reduced NMDA receptor expression,[36] decrease of glutamate-induced pro-apoptotic caspase-3 activation, protection of the survival pathway phosphoinositide-3-kinase/protein kinase B/Akt/glycogen synthase kinase 3 beta, and decrease of neurotoxic inflammation.[35]

Irbesartan has an anticonvulsive effect because decreasing the severity of convulsion and decreases the cell's damage during an acute attack; this effect may be due to blocking of NMDA receptor[28]

Diazepam versus Irbesartan

Comparison between (diazepam) and the tested drug (irbesartan), showed a little difference in some markers expression using IHC, in which, caspase-3 and NMDA had slightly less expression in irbesartan treated group than diazepam treated and this refer to that the irbesartan was more efficient as anti-apoptotic and NMDA receptor blocker than diazepam (Table 3 Figure 1-4).

The behavioral effect in this research showed that irbesartan slightly more anti-convulsant effect than diazepam and decrease seizure potency and duration more than diazepam. Further experimental studies are needed to confirm the anticonvulsant effect of the drug in other models of seizure.

Correlations between IHC scores of Oxidative Stress and Anti-Apoptotic Markers

Correlation between Caspase and Neu N and NMDA-R

Caspase-3 was shown to be a strong positive correlation with NMDA-R, as shown in Figure 5, and these results are in agreement with Bonfoco, 1995 that showed that ionotropic

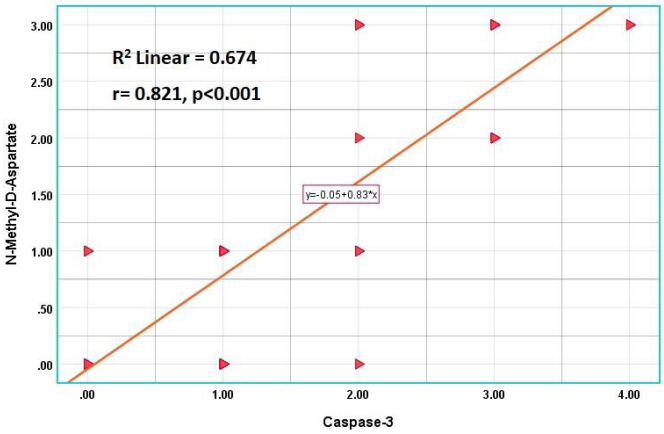

Figure 5: Spearman correlation between caspase-3 and NMDA IHC score

glutamate receptor agonists such as NMDA, induced apoptosis in cultured neurons.[37]

This significant positive correlation gives an idea about the pathway used by the cell to inter the apoptosis process, which is an intrinsic pathway as the expression of the markers increased in a direct proportion manner.

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