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Impact of melatonin against pilocarpine incited seizures in rats

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Abstract

Epilepsy is a standout amongst the most widely recognized genuine mind issue, can happen at all ages and have numerous potential causes. Epilepsy takes place because of a wide range of cell or biochemical changes, for example, modifications in particle channels work, synapse level (excitatory and inhibitory), synapse receptor work, vitality digestion and oxidative stress. This study was performed to explore the possible antiepileptic effect of Melatonin against pilocarpine-induced seizure in male rats. The research was carried out on (40) healthy male Wister rats weighing between 200-300 gm; they were equally allocated to four groups (10 rats in each group).Group (1) normal group (not received any drug), Group (2) negative control group (received only pilocarpine during induction of seizure, Group (3) positive control group (Valproic acid group received 20 mg/kg orally twice daily) and Group (4) Melatonin group (3 mg/kg received orally once a day).Rats of each group (except normal group) were injected intraperitoneal with pilocarpine hydrochloride (400 mg/kg) after 21 days of tested drugs administered orally. The mean onset and duration of seizure were determined to evaluate the efficacy of tested drugs and to compare these effect with that of the normal group and Valproic acid group. Besides, the mean of onset and duration of seizure, neuroprotective effect (Neu N), NMDA receptor, Sodium channels were measured in all groups after convulsion had been induced to detect the effects of the tested drugs on these parameters by comparing them with normal, negative and positive groups. Melatonin had a preventive and anticonvulsant effect against pilocarpine-induced seizure in rats due to decreasing the onset and severity of seizure this effect may be by blocking sodium channels and NMDA receptor also Melatonin had a neuroprotective effect by preventing damage to neurons this effect by decreasing the inflammation and oxidative stress.

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INTRODUCTION

Epilepsy and seizures issue influence 50 million individuals around the globe and add to dismalness and mortality (Amudhan *et al.*, 2015). The utilization of antiepileptic medications is constrained because of the huge swath of unfriendly impacts. For example, subjective impedance, successful disarranges and repeating seizures (Fadheel *et al.*, 2018a). Henceforth, there is a requirement for the improvement of new antiepileptic drugs with less antagonistic impacts and high efficacy. Mela-

tonin actuating one of two pharmacological receptors; MT1 or MT2. These are both G-protein coupled layer receptors, modest quantities (Luther, 2014) of melatonin move the circadian clock prior, in this manner advancing prior rest beginning and morning arousing (Fadheel *et al.*, 2018b). The point of present examination: To contemplate the conceivable antiseizure impact of Melatonin in the aversion of pilocarpine actuated seizure in rodents and to investigate conceivable instrument of activity of Melatonin in immunofluorescent recolouring of NeuN, Nav1.6, and NMDA receptor in rodent mind tissue segments.

MATERIALS AND METHODS

Animals

Forty male Wister rodents (200–300 g) were obtained from the Animal House at the College of Medicine/AL-Nahrain University in Baghdad. The creatures were housed under temperature, mugginess and light-controlled conditions. Every single creature convention was affirmed by the Institutional Review Board at the College of Medicine/AL-Nahrain University. In addition, they were sustained standard oxide sense of taste with water not obligatory.

Pilocarpine-induced status epilepticus

The Pilocarpine-initiated status epilepticus methodology was executed as recently portrayed by (Mundey *et al.*, 2005). Status epilepticus was characterized as a period of consistent seizures that went on for in any event 5 min or seizures that repeated at short interims (<1 min) setting up a continuing epileptiform condition (Beghi *et al.*, 2006). Every single consequent test was performed in the intense period of pilocarpine-initiated epilepsy. Seizures (generalized limbic seizures with status epilepticus) were initiated by a solitary intraperitoneal administration of pilocarpine hydrochloride 4% (400 mg/kg) (Schauwecker, 2012).

Pretreatment experiments

Four groups of rats were utilized for the pretreatment tests (Each group contained 10 rats were taken standard and Melatonin for 21 days orally).

Group 1-(Normal group)

This group not received any drug was served as a normal control group to detect the normal values.

Group 2-(Pilocarpine induced epilepsy group only)

were taken pilocarpine injection intraperitoneal (400mg/kg) considered as epileptic control.

Group 3-(Valproic group)

They were taken 20 mg/kg twice daily of sodium valproate orally before pilocarpine injection. This group filled in as positive control to look at tried groups.

Group 4-(Melatonin group)

They were taken Melatonin 3 mg/kg/day orally before pilocarpine injection

Parameters

After giving the pilocarpine, each rat was cautiously assessed by identifying the onset of the first seizure, duration of seizure, recurrent of seizures and death, recorded by naked eyes; other parameters include the sodium current through sodium channels, the activity of NMDA receptors, Neuro N (a measure of neural cell death).

Immunohistochemistry

Test principle

This procedure established on the recognition of the antigen using specific rat antibodies that binds to specific targeted protein. The assured primary antibody then perceived by secondary antibody (anti-rat) conjugated with biotin, the secondary antibody is then sensed by streptavidin conjugated with horseradish peroxidase polymer which catalyzes the substrate H_2O_2 into unrestricted oxygen and water (H_2O). The unrestricted oxygen then oxidizes 3, 3‘- diaminobenzidine (DAB) into gloomy brown precipitate. A positive reaction will have specified as a brown-coloured precipitate at the antigen site counterstained with hematoxylin and tested for immunoreactivity (Kalyuzhny, 2016).

Immunohistochemical Procedure

These means incorporate planning of slides for recolouring steps; this can be practised by the accompanying advances:

Slide readiness

Paraffin installed areas were cut into 5μm thickness, at that point, the segments were conveyed by cement decidedly charged slides, segments were left to dry to encourage attachment between the segment and the charged glass surface.

Deparaffinization and rehydration

1. Dewaxing of paraffin implanted areas was set inside the sight-seeing oven at 65°C for 30 minutes.
2. Deparaffinization was finished by submerging the slides in xylene for 5 minutes then in crisp xylene for 5 minutes.

3. Rehydration of tissue segment achieved through submerging of slides in successive weakening of ethanol as the accompanying request: firstly use absolute ethanol for 5 minutes, then 95% ethanol used for 5 minutes, then 90% ethanol used for 5 minutes, then 70% ethanol for 5 minutes, 50% ethanol for 5 minutes, and finally distilled water for 5 minutes.

Peroxidase square

Slide circled with Pap pen. Hydrogen peroxide was connected to cover the tissue and brooded for 20 minutes. At that point, the slides were washed with refined water, depleted and blotched delicately

Protein hindering of Non-explicit official of essential immune response

Before including the essential antibodies, slides were prepared for blocking venture, to square endogenous Fc receptor, hatch areas for 20 min to avert any unspecific authoritative of essential counteracting agent (FC district) with tissue segment, this with anticipate false positive outcomes at that point slides were depleted and blotched without washing.

Valuation of the Immunostaining

Valuation of IHC grades for accomplished by light microscope (Genex 20, America) at 40X objective lens with a total power of magnification 400X. The typical results of immunochemical staining found in entorhinal cortex (EC). All results counted as a relative percentage of positive cells stained with dark brown colour out of the total count of positive and negative cells (Shao *et al.*, 2016).

Statistical analysis

The statistical investigates were accomplished with GraphPad Prism® 7.0e (USA). The values are presented as the mean ± standard deviation of the mean. The data were evaluated by means of one-way analysis of variance (ANOVA) after that an LSD Post Hoc to identify significant differences between tested drug group with each normal control, negative control and positive control group. The proportions of the frequency of seizure and mortality rate were described as count and percentage. Statistical significance was demarcated as $P \leq 0.05$ (Blanca *et al.*, 2017).

RESULTS AND DISCUSSION

1-onset of seizure

After pilocarpine injection, the mean onset of convulsion of Melatonin group was (19.5±7.71) minutes, as shown in Table 1.

According to the onset of a seizure in the current study, Melatonin has a significant effect (p£0.05) when likened with negative control whereas non significant difference (p>0.05) when linked with Valproic acid as mentioned in the Figure 1.

2-Duration of seizure

After pilocarpine injection, the mean duration of convulsion of Melatonin group was (5±1.49) seconds, as mentioned in Table 2.

According to the duration of seizure in the present study, Melatonin has highly significant decline (p£0.001) when matched with negative control also highly significant decline (p£0.001) when matched with Valproic acid as shown in Figure 2.

3-Neu N

After pilocarpine injection, the mean of Neu N of Melatonin group after convulsion was (73.6±6.29%) as presented in Table 3.

As per the Neuron antigen in present examination, Melatonin has profoundly critical diminishing (p£0.001) when contrasted and typical control and exceedingly huge increment with negative control yet noteworthy abatement (p£0.05) when contrasted and positive control as presented in Figure 3 and Figure 4

4-NMDA receptors

After pilocarpine injection, the mean of NMDA of Melatonin group after convulsion was (9.8±5.03%) as mentioned in the Table 4.

As indicated by the NMDA receptor in present examination, Melatonin has nonsignificant increment (p>0.05) when weighed and normal control and Valproic acid whereas exceptionally important decline (p£0.001) when weighed with negative control as presented in Figure 5 and Figure 6

5-Nav 1.6

After pilocarpine injection, the mean of Nav1.6 of Melatonin group after convulsion was (10.5±5.46%) as presented in Table 5.

According to the Nav1.6 in present study, Melatonin has significant difference (p£0.05) when related with normal control and highly significant difference (p£0.001) when related with negative control, on the other hand nonsignificant difference (p>0.05) when compred with Valproic acid group as mentioned in Figure 7 and Figure 8

Epilepsy is one of the most widely recognized neurological issues everywhere throughout the world, being related with the paroxysmal release of cerebral neurons and is portrayed by a few side effects

Table 1: Influence of Melatonin on the Onset of Seizure compared with Negative control and Valproic acid in Pilocarpine induced Seizure in rats (n= 10/group)

	Negative	Valproic acid	Melatonin
Onset of seizure	9.6±2.12 minutes	27±8.11minutes	19.5±7.71minutes
Negative		<0.001**	0.006*
Valproic acid			0.070 NS

Data presented as Mean±SD, NS: None statistical significant difference (p>0.05), *:Statistical significant difference (p£0.05), **: Highly statistical significant difference (p£0.001).

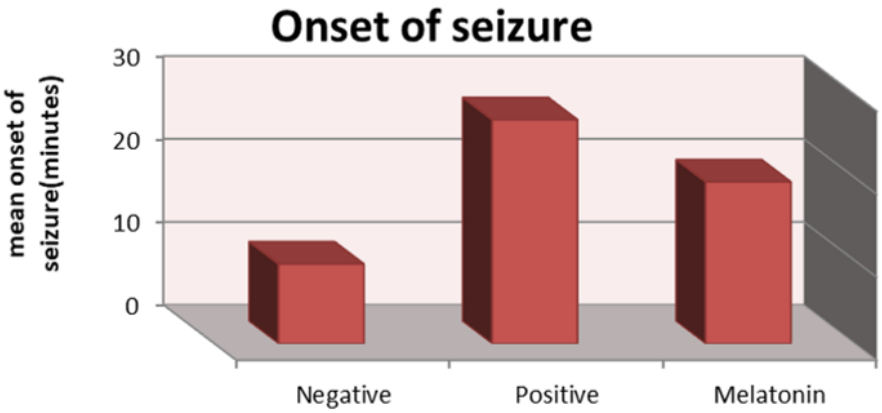

Figure 1: Effect of Melatonin on the Onset of Seizure compared with Negative control and Valproic acid in Pilocarpine induced Seizurein rats (n= 10/group)

Table 2: Effect of Melatonin on the duration of Seizure compared with Negative control and Valproic acid in Pilocarpine induced Seizure in rats (n= 10/group)

	Negative	Valproic acid	Melatonin
Duration of seizure	26.5±3.5seconds	9.3±2.21seconds	5±1.49 seconds
Negative		<0.001**	<0.001**
Valproic acid			0.001**

Data presented as Mean±SD, NS: None statistical significant difference (p>0.05)
*: Statistical significant difference (p£0.05).
**: Highly statistical significant difference (p£0.001).

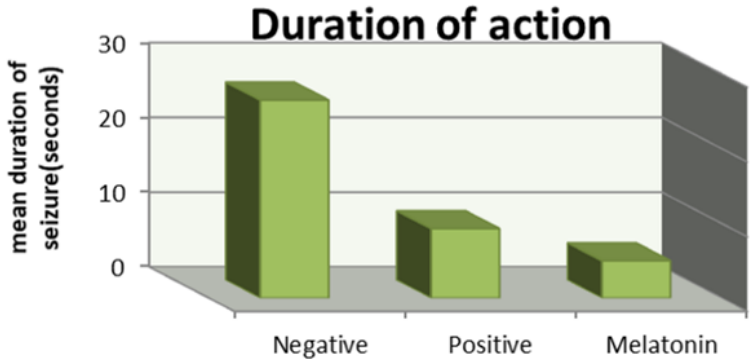

Figure 2: Effect of Melatonin on the duration of Seizure compared with Negative control and Valproic acid in Pilocarpine induced Seizure in rats (n= 10/group)

Table 3: Effect of Melatonin on the neuron cell compared with normal control, Negative control and Valproic acid in Pilocarpine induced Seizure in rats (n= 10/group)

	Normal	Negative	Valproic acid	Melatonin
Neu N	100±0%	62.1±8.54%	83.5±6.64%	73.6±6.29%
Normal		<0.001**	<0.001**	<0.001**
Negative			<0.001**	<0.001**
Valproic acid				0.011*

Data presented as Mean±SD, NS: None statistical significant difference (p>0.05).
*: Statistical significant difference (p£0.05).
**: Highly statistical significant difference(p£0.001)

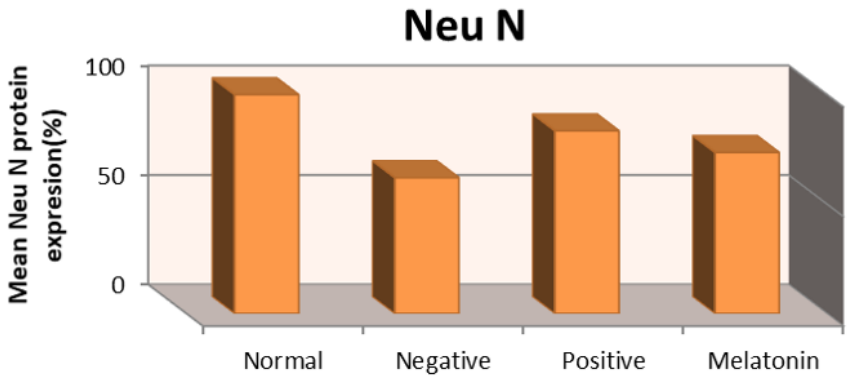

Figure 3: Effect of Melatonin on the neuron cell compared with normal control, Negative control and Valproic acid in Pilocarpine induced Seizure in rats (n= 10/group)

Table 4: Effect of Melatonin on the NMDA receptor compared with normal control, Negative control and Valproic acid in Pilocarpine induced Seizure in rats (n= 10/group)

	Normal	Negative	Valproic acid	Melatonin
NMDAR	3.9±1.2%	33.4±9.5%	9.8±4.69%	9.8±5.03%
Normal		<0.001**	0.132NS	0.132 NS
Negative			<0.001**	<0.001**
Valproic acid				>0.999 NS

Data presented as Mean±SD, NS: None statistical significant difference (p>0.05).
*: Statistical significant difference (p£0.05).
**: Highly statistical significant difference(p£0.001)

Table 5: Effect of Melatonin on the Nav 1.6 compared with normal control, Negative control and Valproic acid in Pilocarpine induced Seizure in rats (n= 10/group)

	Normal	Negative	Valproic acid	Melatonin
Nav1.6	2.1±1.29%	28.5±7.25%	10.4±4.12%	10.5±5.46%
Normal		<0.001**	0.003*	0.003*
Negative			<0.001**	<0.001**
Valproic acid				0.999 NS

Data presented as Mean±SD, NS: None statistical significant difference (p>0.05).
*: Statistical significant difference (p£0.05).
**: Highly statistical significantdifference (p£0.001)

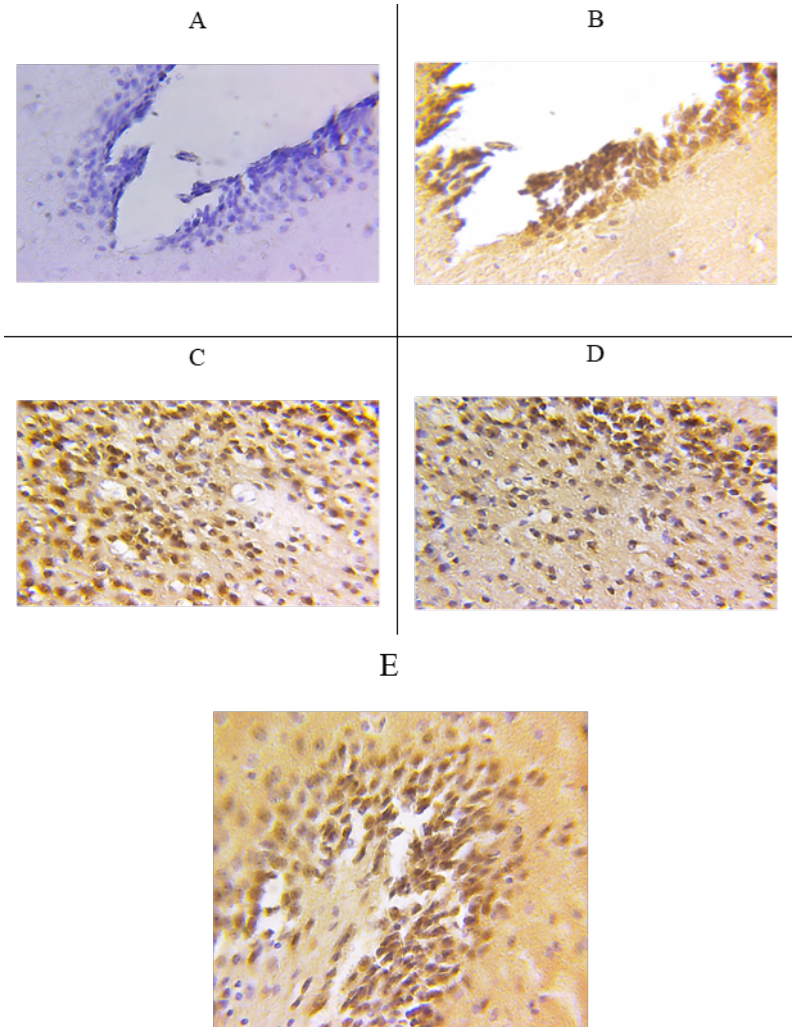

Figure 4: Showing Neu N staining using Rabbit polyclonal anti- Neu N diluted as 10pg/ml visualized by peroxidase conjugate enzyme. (A). IHC quality control (staining without adding primary antibody)showing negative results. (B). Normal rat (without treatment or induction) showing staining of all neuronal cells with intense dark brown color. (C). Negative control (induction only), (D). Positive control (treated with Valproicacid), (E). Melatonin treated

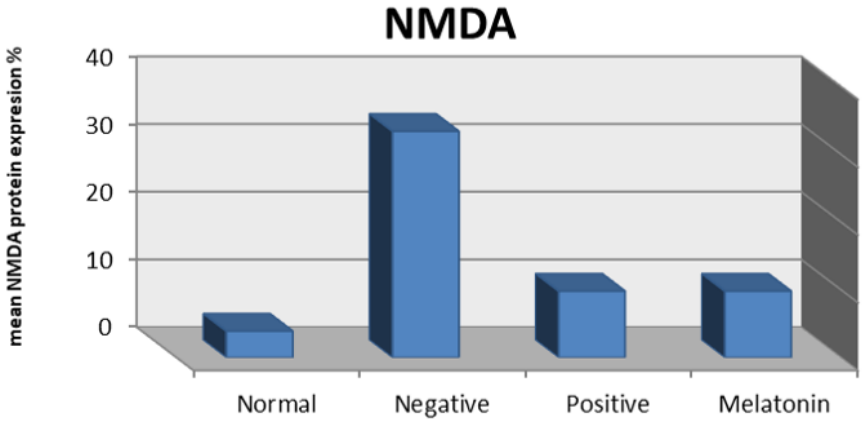

Figure 5: Influence of Melatonin on the NMDA receptor compared with normal control, Negative controland Valproic acid in Pilocarpine induced Seizure in rats (n= 10/group)

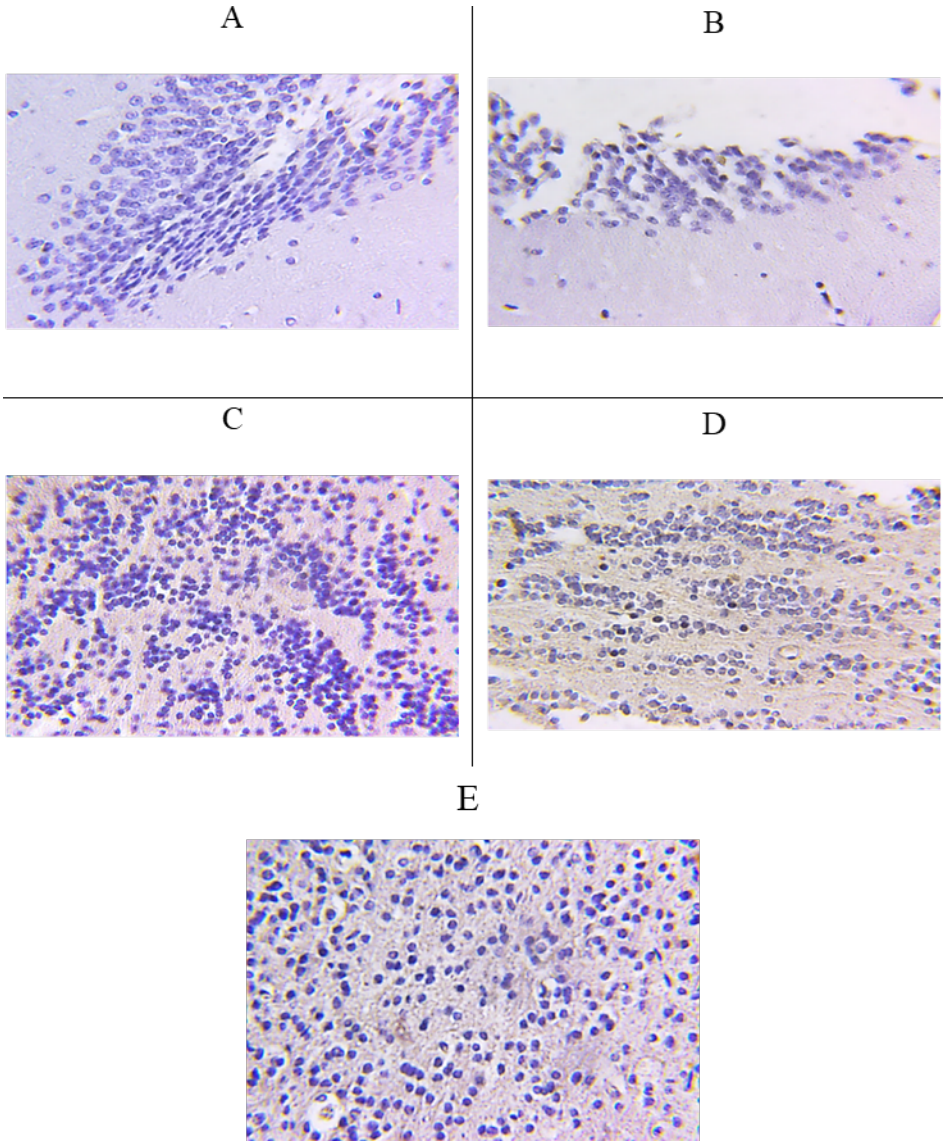

Figure 6: Showing NMDA receptor staining using Rabbit polyclonal anti- NMDA antibody diluted as 10pg/ml visualized by peroxidase conjugate enzyme. (A). IHC quality control (staining without adding primary antibody) showing negative results. (B). Normal rat (without treatment or induction) showing staining of all neuronal cells with intense dark brown color. (C). Negative control (induction only), (D). Positive control (treated with Valproic acid), (E). Melatonin treated

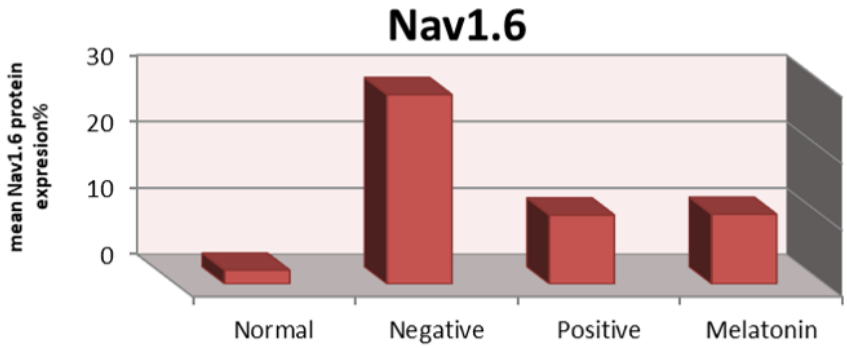

Figure 7: Effect of Melatonin on the Nav 1.6 compared with normal control, Negative control and Valproic acid in Pilocarpine induced Seizure in rats (n= 10/group)

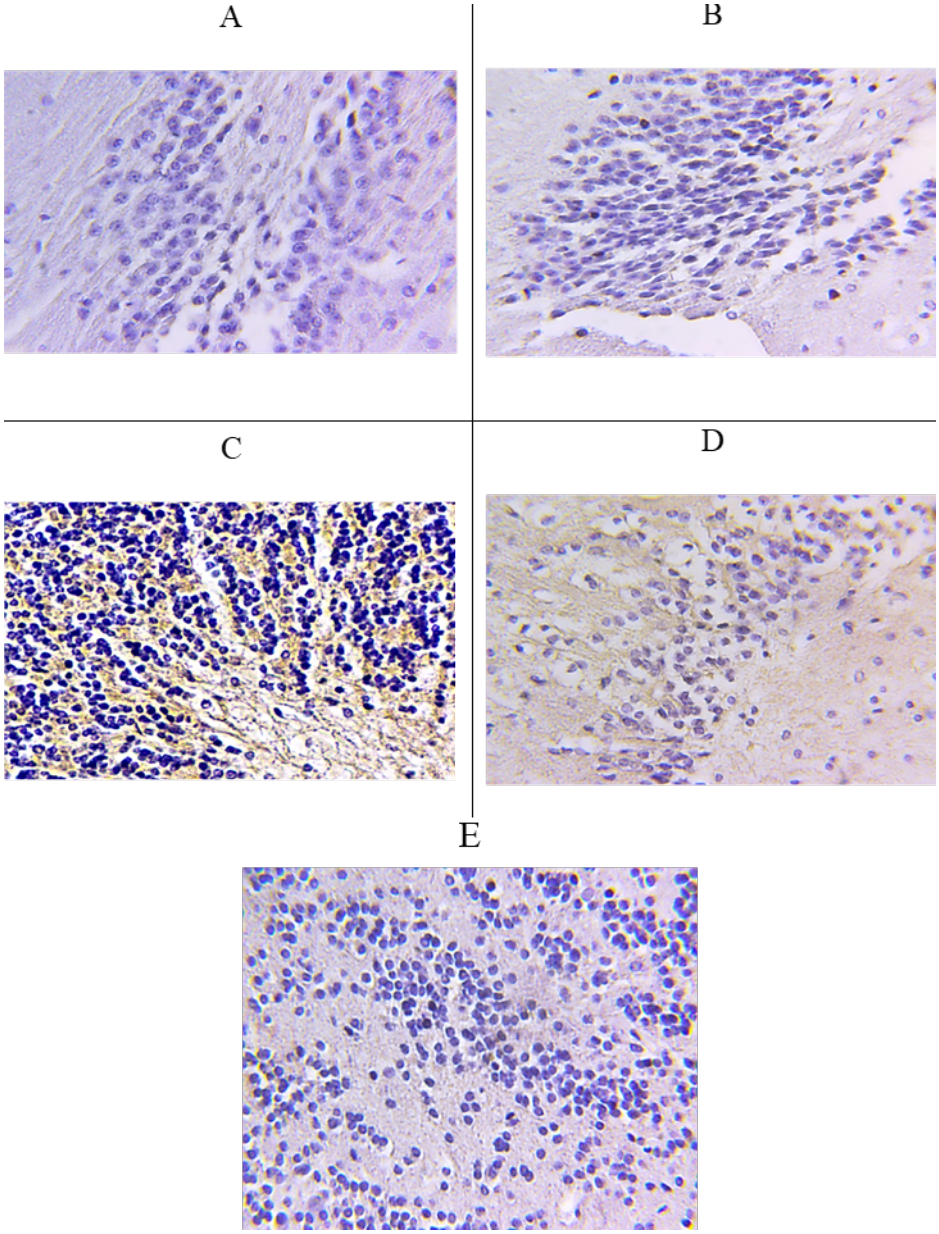

Figure 8: Showing Nav 1.6 receptor stainingusing Rabbit polyclonal anti- Nav 1.6 antibody diluted as 10 pg/ml visualized by peroxidase conjugate enzyme. (A). IHC quality control (staining without adding primary antibody) showing negative results. (B). Normal rat (without treatment or induction) showing staining of all neuronal cells with intense dark brown color. (C). Negative control (induction only), (D). Positive control (treated with Valproic acid) (E). Melatonin treated

including changes of practices and awareness supported modification in cerebrum work (Freitas *et al.*, 2004). The pilocarpine gives a valuable creature model for examining components and restorative ways to deal with epilepsy. In this model, extreme and continued incitement of cholinergic receptors can prompt seizure-related cerebrum harm in rodents (Khongsombat *et al.*, 2010).

Impact of Melatonin

In the present examination, Melatonin has an anticonvulsive impact since reductions the beginning and seriousness of seizure and abatement the phones harm during intense assault this impact because of obstructing of sodium channels and NMDA receptor. (Molina-carballo and Muñoz-hoyos, 1997) announced information demonstrate that melatonin represses cerebrum glutamate receptors and nitric oxide creation, along these lines recommending that it might apply a neuroprotective and antiexcitotoxic impact. Melatonin has been believed to counteract seizures in a few creature models and to diminish epileptic signs in people. Melatonin has demonstrated to be valuable as adjunc-

tive treatment in the clinical control of serious childish myoclonic epilepsy. The outcomes recommend that melatonin may have a valuable job in instruments of neuroprotection and furthermore demonstrated to be utilized in different instances of untreatable epilepsy. Further examinations utilizing more patients and placebo-treatment would be valuable in understanding the potential utilization of melatonin as a co-therapy now and again of seizures. (Borowicz *et al.*, 1999), proposed that the Melatonin before the test essentially brought the electroconvulsive edge up in mice. The defensive activity of melatonin (50 mg/kg) in the electroconvulsive edge test was switched by aminophylline, picrotoxin and bicuculline. Melatonin at the sub convulsive portion of 25 mg/kg potentiated the anticonvulsive movement of carbamazepine and phenobarbital. No potentiation was seen on account of valproate and diphenylhydantoin. Melatonin did not impact the plasma or mind levels of enemies of epileptics examined, so pharmacokinetic communication isn't plausible. Melatonin (25 mg/kg) alone and its mixes with carbamazepine or phenobarbital, giving a half assurance against maximal electroshock. The present investigation as per (Andrabi *et al.*, 2004) who revealed that melatonin diminished the NMDA-instigated supported cytosolic Ca2+ levels. Mitochondria go about as Ca2+ cradles by sequestering abundance Ca2+ from the cytosol. [Ca2+] keeps on rising when NMDA receptors are persistently invigorated, causing Ca2+ take-up into the mitochondria that, after achieving an edge level prompts opening, which thusly delivers Ca2+-initiated Ca2+ discharge. Surely, brought down the NMDA-instigated [Ca2+] c levels, showing the interceded Ca2+ discharge that adds to the general [Ca2+] c rise. Helpful utilization of melatonin may give a procedure to the treatment of stroke and neurodegenerative issue that include the mitochondrial apoptotic pathway. Additionally, the present investigation perfect with (Tchekalarova *et al.*, 2013) notice that Melatonin is a strong cell reinforcement which indicated anticonvulsant exercises both in test and clinical examinations. They inspected the impact of melatonin treatment (10 mg/kg/day, weakened in drinking water, two months) during epileptogenesis on the results of kainate - actuated status epilepticus in rodents. Melatonin expanded the inertness in the presence of unconstrained intermittent seizures and diminished their recurrence just during the treatment time frame. The social changes related to hyperactivity, despondency like conduct during the light stage were decidedly influenced by melatonin treatment in rodents with epilepsy. Also, (Geronzi *et al.*, 2018) detailed that the mind is especially vulnerable to oxidative pressure being the most vigorously dynamic organ in the body because of its high metabolic requests. There is proof that neuronal hyperexcitability and oxidative damage delivered by an intemperate generation of free radicals may assume a job in the inception and movement of epilepsy. Understanding the job of oxidative worry in epileptogenesis is fundamental to depict fitting remedial techniques. Neuroprotectant or antioxidant agent mixes may apply constructive outcomes when related to antiepileptic drugs (AEDs) (Geronzi *et al.*, 2018).

CONCLUSION

Melatonin has an effective defensive effects against seizures made by Pilocarpine in rat which were similar to that of valproate acid.

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