

Research Paper: Hepatocyte Growth Factor Attenuates the Severity of Status Epilepticus in Kainic Acid-induced Model of Temporal Lobe Epilepsy by Targeting Apoptosis and Astrogliosis



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ABSTRACT

Introduction: Although pharmacotherapy is the most common treatment for epilepsy, proper seizure control is not achieved with current medications. This study evaluated the protective effects of the Hepatocyte Growth Factor (HGF) in a rat model of Temporal Lobe Epilepsy (TLE) and explored possible molecular mechanisms.

Methods: A TLE rat model was determined using an intra-hippocampal kainic acid injection (4 µg). Intra-cerebrovascular injection of HGF (6 µg) was performed 30 min before kainic acid injection. Learning and memory impairment were investigated by behavioral tests. The Enzyme-Linked Immunosorbent (ELISA) was used to determine astrogliosis and DNA fragmentation. Changes in neuronal density and mossy fiber sprouting were evaluated by Nissl and Timm staining, respectively.

Results: Behavioral assessments indicated that kainate-treated rats presented spontaneous seizures. Moreover, their alternation percentage scores in the Y-Maze test were lower ($P < 0.001$). Likewise, the passive avoidance test confirmed learning disability in Kainate-treated rats ($P < 0.001$). HGF administration reduced the number of spontaneous seizures, alternation percentage score ($P < 0.001$), and cognitive disturbances ($P < 0.001$). The histopathological results also showed that a protected HGF administration contributed to the reduction of neuronal loss in the CA3 subregion of the hippocampus and inhibited the formation of aberrant Mossy Fiber Sprouting (MFS) ($P < 0.01$). Furthermore, the ELISA data indicated a significant decrease in GFAP ($P < 0.01$) and DNA fragmentation ($P < 0.05$) following HGF administration.

Conclusion: Our findings demonstrated the validity of HGF in protection against the progression of the kainate-induced TLE in rats. This measure improved learning, cognitive disturbances and inhibited apoptosis and astrogliosis.

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Highlights

- Temporal lobe epilepsy results in apoptosis of neuronal cells;
- Hepatocyte growth factor attenuates the severity of status epilepticus in kainic acid-induced model;
- Hepatocyte growth factor attenuates apoptosis of neuronal cells in kainic acid-induced model of temporal lobe epilepsy.

Plain Language Summary

Epilepsy is known as a disorder of the CNS which is caused by an imbalance in the electrical activity of neurons that in turn results in derangement in cognitive or causing debilitating seizures. Hepatocyte growth factor is one of neurotrophins secreted from mesenchymal and epithelial cells that regulate the growth, survival and functional changes of cells through signaling pathways such as the tyrosine kinase pathway after binding to its specific receptor. In this study, we tried to find out the effect of hepatocyte growth factor on attenuation of the severity of status epilepticus in kainic acid-induced model of temporal lobe epilepsy. Our results show that hepatocyte growth factor is able to protect against progression of the kainate-induced temporal lobe epilepsy in rats by improvement of learning, cognitive disturbances and inhibiting of apoptosis and astrogliosis.

1. Introduction

The brain has numerous local inhibitory and stimulatory circuits interacting with each other. In addition to these circuits, each brain region mainly receives stimulatory or inhibitory inputs from other areas. There is a precise balance between these stimulatory or inhibitory circuits (Löscher, Klitgaard, Twyman, & Schmidt, 2013). Generally, stimulatory circuits tend to spread across communicative neurons. However, in normal conditions, they are controlled through inhibitory mechanisms. Thus, any agents that facilitate the transmission of stimuli or prevent the transmission of inhibitory factors give rise to epilepsy. In general, epileptic seizures occur with the loss of ionic fluid balance in either glutaminergic or inhibitory stimulus synapses. Temporal Lobe Epilepsy (TLE) is among humans' most common focal seizures and a chronic neurological disorder that affects approximately 50 million individuals worldwide. Its main feature is spontaneous seizures (Cano et al., 2018).

There is some dissimilarity between animals and humans in temporal lobe epilepsy. Numerous TLE patients display an atrophic hippocampus; however, a possible source of spontaneous seizures, animals with TLE exhibit a type of hippocampal damage. It is inconsistent with humans and often involves a minor part of a much greater constellation of damage to other brain structures. Likewise, TLE patients display developmental structural abnormalities, i.e., the key symptoms and signs in clinical etiology, while severe insults in initially laboratory

rats have been reported (Sloviter, 2005). The preservation of GABA-ergic inhibitory interneurons and neurogenesis are fundamental issues that need much attention in the treatment of epilepsy.

Epilepsy indicates brain dysfunction, and multiple pathologic factors can cause it. Accordingly, different therapies are considered to improve epileptic seizures, partly based on neurotransmitters' involvement. Owing to shortcomings and intolerable side effects of anti-epileptic drugs, there is an urgent need to develop new therapeutic agents and approaches to treat this disease (Asadi Pooya, Stewart, Abrams, & Sharan, 2017). A study reported that reducing Hepatocyte Growth Factor/Scatter Factor (HGF/SF) can significantly decrease the numbers of neocortical GABAergic interneurons and spontaneous seizures (Bae et al., 2010). It is well documented that Hepatocyte Growth Factor (HGF) expression in the prenatal forebrain contributes to neuronal migration by its protease activity. Loss of receptors, such as Plaur for HGF activators, like urokinase-type plasminogen activator, gives rise to the attenuation of HGF/SF in the embryonic forebrain, interneuron deficits, and finally, spontaneous seizures (Bae et al., 2010). The reduced number of neocortical GABAergic interneurons and their apoptosis can be linked to derangement in the activity of Dlx homeodomain transcription. It is an essential factor in developing the production of forebrain GABAergic interneurons (Cobos et al., 2006).

HGF is among the neurotrophins secreted from mesenchymal and epithelial cells that regulate growth, surviv-

al, and functional changes through signaling pathways, such as the tyrosine kinase pathway, after binding to its specific receptor (c-MET) (Molnarfi, Benkhoucha, Funakoshi, Nakamura, & Lalive, 2015). Studies indicated that the mice with a mutation in the plasminogen urokinase gene (which kinase plays an essential role in the expression and signaling pathway of HGF) indicated a 50% reduction in HGF levels and a substantial decrease in GABA inhibitory interneurons, indicating high susceptibility of mice to epilepsy (Powell, et al., 2003; Simonato, Tongiorgi, & Kokaia, 2006). Furthermore, a substrate for GABA production in inhibitory GABAergic neurons is glutamine. The loss of glutamine synthesis in reactive astrocytes leads to a decrease in GABA production and subsequent initiation of apoptosis (Devinsky, Vezzani, Najjar, De Lanerolle, & Rogawski, 2013). Moreover, increased endogenous HGF levels in epileptic mice caused by pentylentetrazole increased GABA inhibitory interneurons (Bae et al., 2010). Based on the above reports, we sought to determine whether HGF can reduce seizure severity and improve learning by targeting astrogliosis.

2. Methods

Forty adult male Wistar rats weighing 200-250 g were obtained from the Experimental Studies Center of Iran University of Medical Sciences. Animal Ethical Committee of Iran University of Medical Sciences approved all the experiments and protocols. Furthermore, all efforts were made to minimize suffering. Rats were kept at a controlled temperature room (22-24 °C) with 55% humidity under a 12:12 h light/dark cycle, with access to food and water ad libitum.

Animals were randomly divided into four experimental groups: sham (n=10), kainic acid (n=10), epilepsy + HGF (6 µg) (n=10) group, epilepsy+valproic acid (200mg/kg IP) (n=10). Rats were anesthetized with ketamine (100 mg/kg, IP) and xylazine (5 mg/kg, IP). The necessary injections were performed with the stereotaxic method. In the Kainic acid group, the animals received unilaterally 0.4 µg/µL of Kainic acid (Sigma Chemicals, USA) with 10 µL saline in the posterior part of the hippocampus. HGF (Sigma Chemicals, USA) was dissolved in 30% Cromophor and injected 30 min before Kainic acid injection 6 µg; ICV). To investigate the seizure status of Kainic acid, Racine scoring was used: Number 0: No response; Number 1: Facial and mouth movements; No. 2: Up and down movements of head; Number 3: Clonus movements in the anterior limb; Number 4: Lifting on two legs with Clonus of the two anterior limbs; No. 5: Lifting on two legs and losing balance.

Behavioral studies

Y-Maze test and alternation behavior

In this test, the performance of animals in terms of working memory by observing and measuring the alternation spontaneous behavior of the animal is examined in a working session. The maze of this test is made of plexiglass, and each arm has dimensions of 40×30×15, and the arms are connected through a central area. To conduct the test, each rat is placed at the end of the arm, and its free access is allowed to all areas of the maze at a time interval of 8 min. The number of times the animal enters into each arm is recorded by observation. Spontaneous alternations were counted when the animal's hind legs were wholly inserted into the arm. Alternation behavior is considered as successful and serial entries in all the arms in the triple sets. Thus, the percentage of the alternation is calculated based on the following formula: [(actual alternation)/(maximal alternation-2)]×100. The test was performed by an examiner blinded to the experimental design.

Passive avoidance test

A shuttle box was used to evaluate Passive Avoidance Learning (PAL) two days after the Y-Maze test. It is made from a two-part plexiglass box with a bright part and a dark part, and the dimensions of the two parts are equal (20×20×40 cm). There are stainless steel bars on the floor of both parts with a 1 cm distance. A 100-W light bulb is placed 40 cm above the device's bright side. There is a guillotine door between two chambers. This test includes the following steps: A) adaptation stage: at this stage, each animal was habituated with passive avoidance apparatus for ≥5 min for two consecutive days before starting the experiment. B) Acquisition stage: at this stage (the third day), the animal is placed in a bright part, and the chamber is kept dark for two min. During this time, the two dark and bright chambers are completely closed via a guillotine door. The chamber's light is turned on at the end of the period, and the guillotine door is opened. Once the door is opened, the chronometer is used. The time it takes for the animal to go from the light chamber to the dark chamber is recorded, i.e., referred to as "Initial latency" (the criterion for the animal entering into the dark is the dorsal motor limbs from the connecting door of two compartments). Then, the door is closed, and a single electric shock (1 mA for 1 second) is given to the animal. The animal is transferred to the cage at 1 min after the end of the work. At this step, rats with a delay of more than 60 s are excluded from the tests. The retention and recall step is performed 24 h after the second stage on the fourth day. This stage is

similar to the previous stage, with the difference that the animal will not receive any shock when it enters the dark chamber. STL (step-through latency) is measured at this point. STL indicates the time the animal remains in the light chamber before entering the dark chamber. Cut-off time is 480 s if the rat does not enter the dark chamber.

Evaluating the GFAP expression

The GFAP expression was determined using commercial-ly available kits based on the manufacturer's instructions.

Histological studies

Nissl staining

At the end of behavioral tests, several animals ($n=5$ /group) were perfused transcardially with PBS and paraformaldehyde in PBS, respectively. Next, the samples were fixed in 10% formalin, then embedded in paraffin, and cut into 7- μ m thickness slices by microtome. Finally, the samples were stained using cresyl violet according to the Nissl standard clinical laboratory protocol. The neuronal cells in the CA3 area of the hippocampus were counted using an optical microscope (Olympus CK2; Olympus Optical Co., Japan). Cells were counted by an examiner blinded to experimental design.

Timm staining

At the end of behavioral tests, a specific number of animals ($n=5$ /group) were perfused via ascending aorta with 0.9% sodium chloride (2 min), 0.37% sodium sulfide (5 min), 0.9% sodium chloride (1 min), 4% formaldehyde in PBS (30 min), respectively. In the next step, the brain tissues were dissected from skull bone and were post-

fixed in paraformaldehyde in PBS for 24h. Then, the samples were fixed in 30% sucrose and cut into 30- μ m thick sections using cryostats on gelatin-coated slides. In the next step, the sections were treated in 120 ml 50% Arabic gum, 20 ml 2 M citrate buffer, 60 ml 0.5 M hydroquinone, and 1 mL 19% silver nitrate. Finally, the sections were incubated with 5% sodium thiosulfate for 5 min after washing.

All data are expressed as Mean \pm SD. Data analysis was performed using GraphPad PRISM 6 software (San Diego, CA, USA). One-way Analysis of Variance (ANOVA), followed by Tukey's post hoc test, was applied to compare three or more groups. $P<0.05$ was considered significant.

3. Results

Seizure Behavior assessment

As per Table 1 and Figure 1, the results of seizure behavior quantity of animals based on the ranking of Racine (ranging from 0 to 5) suggested that the Kainate+HGF group had a significant decrease in rates of spontaneous seizures and seizure intensity, compared to the kainic acid group ($P<0.05$).

Spatial memory survey using Y-Maze test

Two parameters were evaluated in this test:

i) Alternation percentage

As depicted in Figure 2, our results demonstrated a significant difference in alternation percentage between the animals in the sham group and the epileptic group ($P<0.001$). The rate of alternations in the kainate-treated

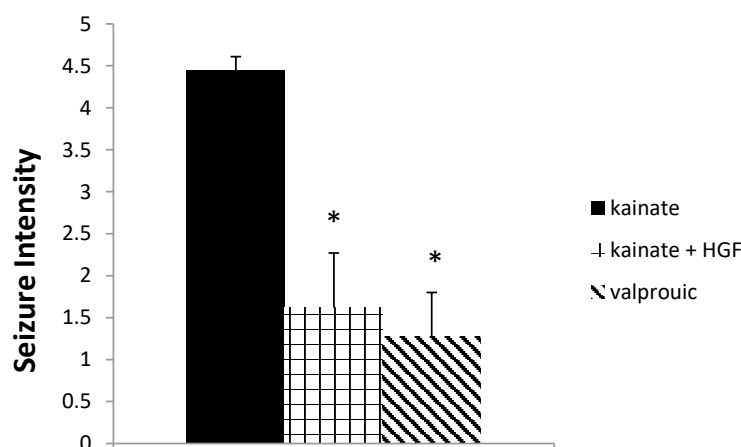


Figure 1. Seizure scores were significantly reduced following pre-treatment with HGF

* $P<0.05$ vs. Kainate group.

Table 1. The numbers and rates of spontaneous seizures per group

Groups	No. (%)	
	Class 4 Seizure	Class 5 Seizure
Sham	0(0)	0(0)
Kainate	6(60)	4(40)
Kainate+HGF	2(20)	0(0)
Kainate+valproate	0(0)	0(0)

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rats was significantly lower than that of the sham-operated rats. A significant increase in alternation percentage was observed in Kainate+HGF group, compared to only the kainate-treated rats ($P<0.001$). Furthermore, the administration of valproic acid increased the alternation percentage of the kainate+valproic acid group, compared to only the kainate-treated rats ($P<0.001$). There was no significant difference between Kainate+HGF and kainate+valproic acid groups.

ii) The total number of arms that the animal entered in 8 min

Our results revealed no significant differences between the mean number of arms the animals entered in different groups (Figure 3).

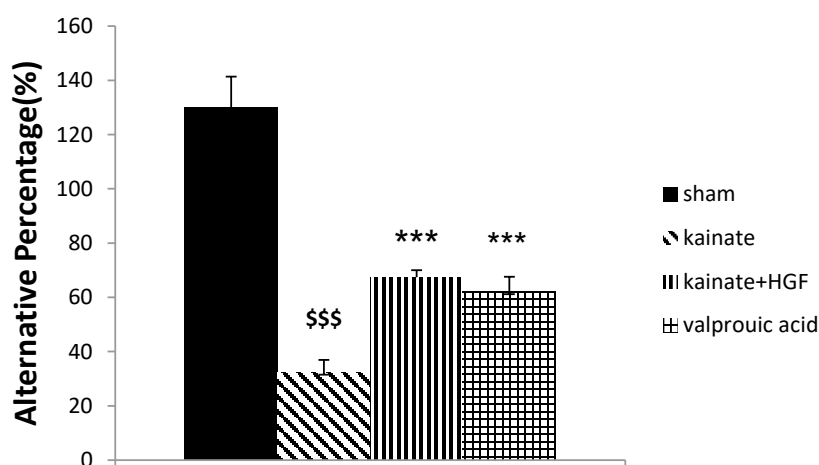
iii) Investigating passive avoidance behavior using shuttle box

As per Figure 4-A, our results presented no significant differences in Training Latency in the studied groups

Moreover, our statistical analyses indicated that the kainate-treated rats significantly decreased STL compared to the sham group ($P<0.001$). There was a significant difference in STL between only the kainate-treated rats and the kainate-treated rats that received 6 µg of HGF 30 min before the injection of kainic acid ($P<0.001$). Additionally, the administration of valproic acid resulted in a significant increase in STL compared to the kainate-treated rats ($P<0.001$) (Figure 4-B).

Evaluating the effects of HGF on astrogliosis

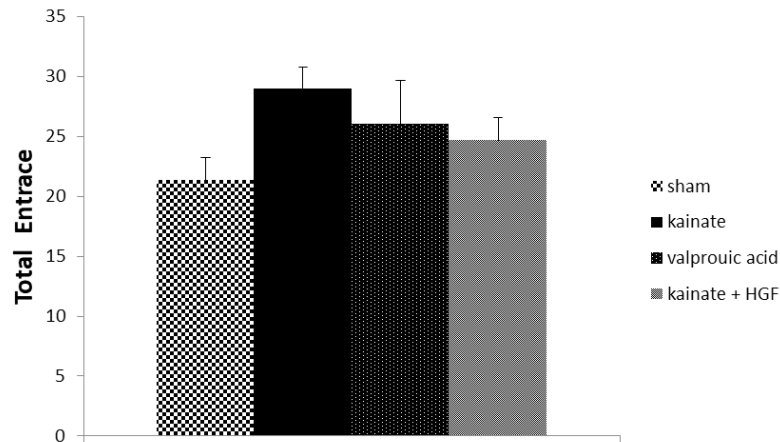
The measurement of GFAP was performed as a specific marker for the study of astrocytes and astrogliosis changes in hippocampal tissue. ELISA data demonstrated that the expression of GFAP was significantly elevated in the kainate-treated rats compared to the sham animals ($P<0.05$). Pre-treatment with HGF markedly



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Figure 2. The frequency of alternations was significantly increased following pre-treatment with HGF

\$\$\$ $P<0.001$ vs. Sham; *** $P<0.001$ vs. Kainate group.



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Figure 3. Pre-treatment with HGF did not affect the mean of the total number of arms the animals entered in experimental groups

reduced the expression of GFAP, than the kainic acid group ($P < 0.01$). Pre-treatment with HGF markedly decreased the level of GFAP, than the kainic acid group; however, there were significant differences between the sham and Kainate +HGF groups (Figure 5).

Evaluating the effects of HGF on DNA fragmentation

To estimate the effects of HGF on apoptotic cell death, the amount of DNA fragmentation as an indicator of apoptosis was determined. As shown in Figure 6, a comparison of the kainate group with the sham group suggested a significant increase of this parameter in the kainate-treated

rats ($P < 0.01$). Our results also indicated that pre-treatment with HGF significantly reduced DNA fragmentation compared to the only kainate-treated rats ($P < 0.05$). Moreover, there was a significant decrease in the kainate+valproate group compared to the Kainate group ($P < 0.01$). There were no significant differences among the sham, Kainate +HGF, and kainate+valproate groups.

Evaluating neuronal density in the CA3 subregion of the hippocampus

In the histopathologic study by Nissl staining method and the use of Cresyl Violet in the sham group, in the CA3

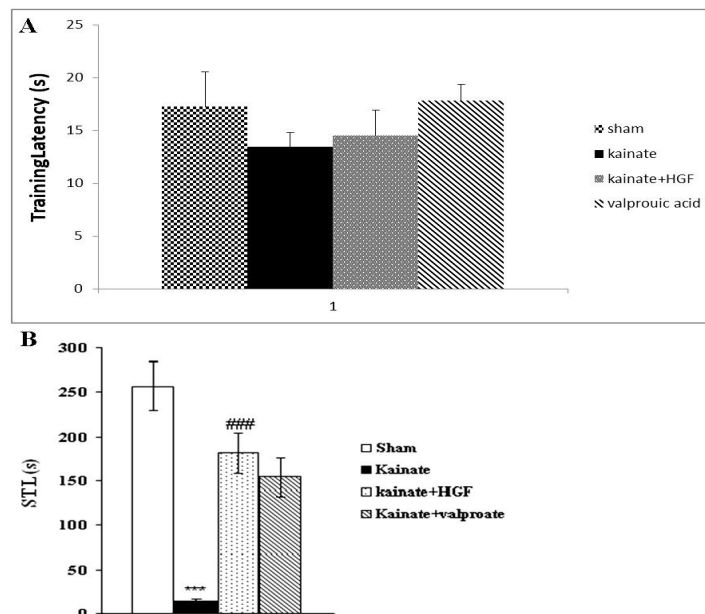
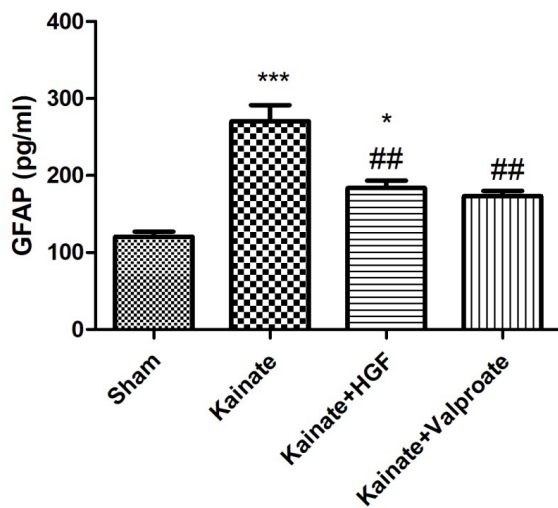


Figure 4. The effects of HGF on latency training and STL

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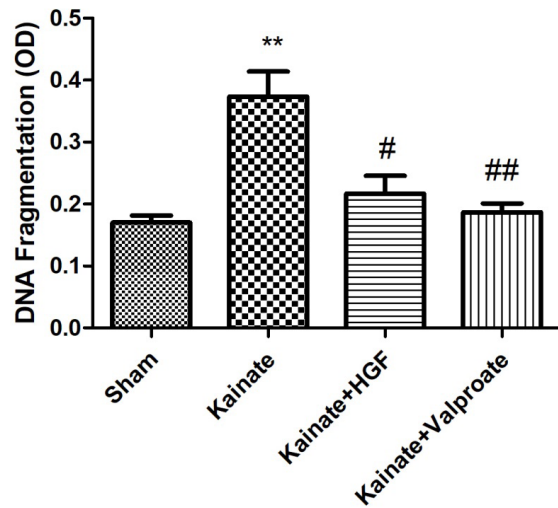
A and B: Pre-treatment with HGF did not affect Training Latency whereas significantly improved STL, compared with the kainite group. *** $P < 0.001$ vs. Sham; ### $P < 0.001$ vs. Kainate group.



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Figure 5. Pre-treatment with HGF significantly reduced the expression of GFAP as a specific marker for the study of astrogliosis
*** P<0.001 and * P<0.05 vs. Sham; ## P<0.01 vs. Kainate group.

region of the hippocampus, the neurons with round, prominent nuclei and clear cytoplasm were visible (Figure 7). A significant decrease in neuronal density was observed in the CA3 subregion of the hippocampus in the kainate-treated rats (P<0.01). Treatment with the HGF and valproic acid groups markedly increased neuronal density, compared to the Kainate group (P<0.05).

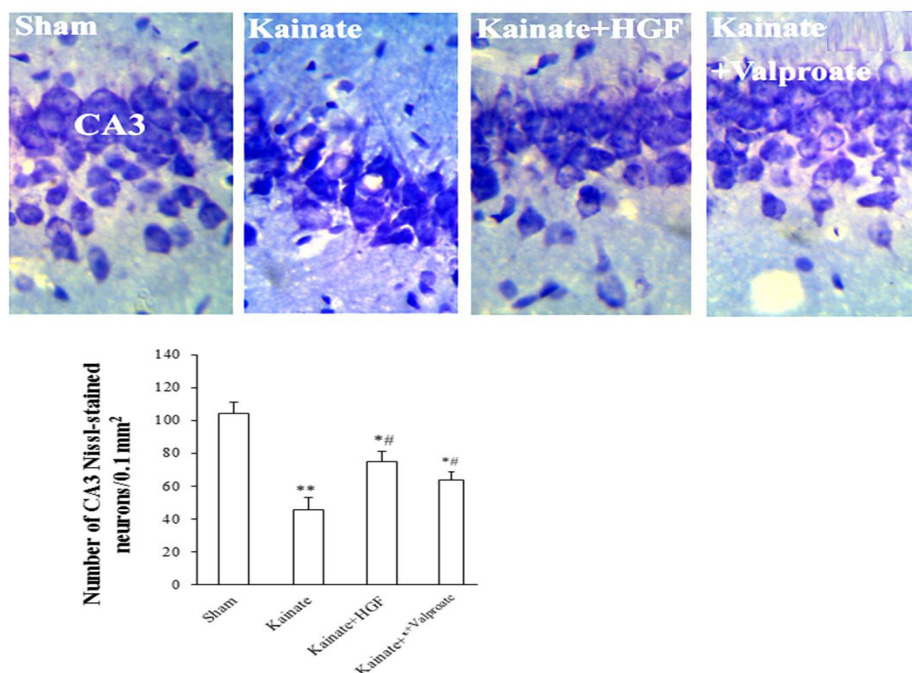


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Figure 6. Pre-treatment with HGF significantly reduced DNA fragmentation as an indicator of apoptosis
** P<0.01 vs. Sham; # P<0.05 and ## P<0.01 vs. Kainate group.

Evaluating mossy fibers sprouting in the CA3 subregion of the hippocampus

Timm staining with silver nitrate was used to study the mossy fibers sprouting intensity in the dentate gyrus of the hippocampus (Figure 8). Accordingly, this index was significantly increased in the kainate-treated



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Figure 7. Pre-treatment with HGF significantly improved neuronal density in the CA3 subregion of the hippocampus
** P<0.01 vs. Sham; # P<0.05 and ## P<0.01 vs. Kainate group.

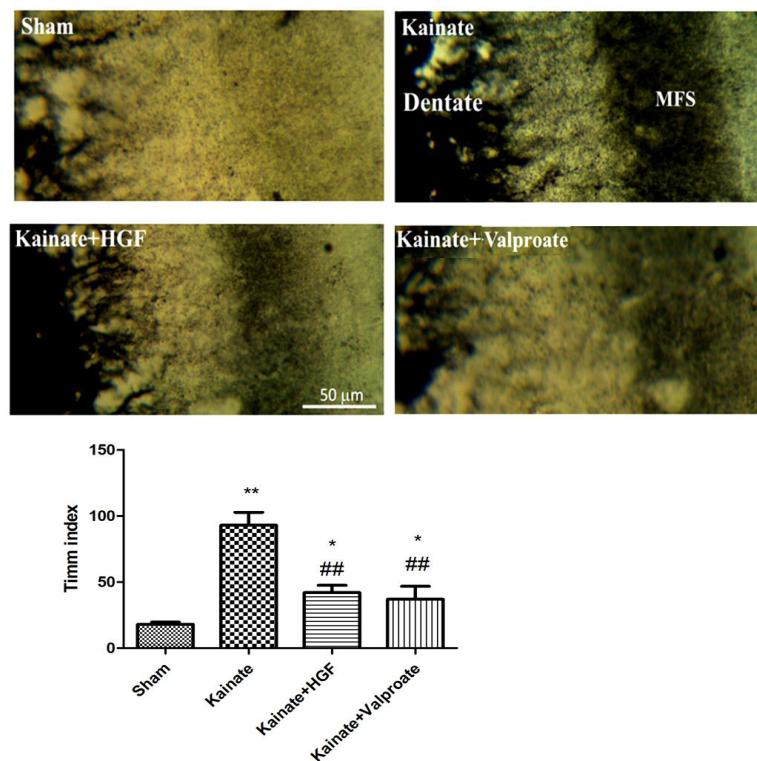


Figure 8. The optical microscope images of hippocampus dentate gyrus stained by the Timm staining. Pre-treatment with HGF significantly prevents the development of aberrant mossy fibers sprouting (** $P < 0.01$; and * $P < 0.05$ vs. Sham; ## $P < 0.01$ vs. Kainate group).

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rats, compared with the sham animals ($P < 0.0001$). A significant decrease in the mossy fibers sprouting intensity was found following HGF and valproic acid ($P < 0.01$). However, the values of this parameter in the groups mentioned above still had a significant difference with the sham group ($P < 0.01$).

4. Discussion

Applying HGF resulted in decreased astrogliosis and GFAP responses in the present study. There exists no similar study in this field; however, this result was predictable due to the role of this neurotrophin factor in protecting the GABA-ergic inhibitory role of these interneurons in protecting astrocytes (Bae et al., 2010). In agreement with our findings, a study indicated that HGF-preconditioned neural progenitor cells promoted neurite outgrowth by attenuating astrocyte reactivity (Dragas, 2018). Moreover, using HGF in this study increased neuronal density in the hippocampus CA3 region. Earlier studies also presented that HGF can play a crucial role in synaptic plasticity in hippocampal neurons through its signaling pathways (Sharma, 2010). Sun.W et al. also revealed that using HGF can improve neuronal damage in

the ALS model and increase the duration of neuronal life (Sun, Funakoshi, & Nakamura, 2002).

Implementing HGF also significantly reduced the Timm index in this study, indicating a decrease in the aberrant mossy fiber sprouting in the hippocampus CA3 region. There is no similar study in this regard; however, in studying the role of other neurotrophins in epilepsy, the NT-3 in vivo injection has resulted in suppressing the supraventricular sprouting of mossy fibers caused by kainic acid (Xu, Michalski, Racine, & Fahnestock, 2002).

In our study, using HGF also significantly reduced DNA damage compared to the Kainate group. In previous studies, the anti-apoptotic function of HGF was determined by controlling Caspase 3 and the induction of Bcl2 (Yamamoto et al., 2001). Additionally, a study demonstrated that HGF could exert anti-apoptotic effects in a mouse model of acute liver failure as well as on in vitro human hepatocyte injury (Motoi et al., 2019).

Overall, the intra-cerebrovascular injection of HGF can benefit both initial kainic acid-related seizures and seizures related to resistant epilepsy after 21 days. Previous studies suggested the effects of HGF on GABA inhibitor

interneurons. Identifying uPA as part of the HGF signal pathway relevant to GABA interneurons reinforces the notion that HGF can prevent resistant epilepsy (Bottaro et al., 1991). In the study of HGF-infected rats without uPA, Powell et al. observed GABA-ergic interneurons increase and subsequently improved seizure-induced epilepsy (Powell et al., 2003).

Aguilar et al. also argued that 8 g/kg HGF injection subcutaneously for 7 days before kainic acid injection to mice resulted in increased neurons and GABA-ergic activity. Consequently, the stimulatory effects of kainic acid were reduced. Although no histological tests were performed in their study, by investigating Racine scoring and observing its tangible reduction, the impact of kainic acid proved to be effective. The decrease in brain waves strength, alpha, beta, and gamma indicated improved GABA-ergic activity. These results are entirely consistent with our behavioral outcomes.

Concerning epileptic behaviors caused by the kainic acid injection, studies indicated that kainic acid injection leads to premature seizures in the early 4 to 5 hours, which can be evaluated by Racine scoring (Racine, 1972). Likewise, our study suggests that kainic acid injection causes kainic seizures in the first 4 hours after induction. Injection of HGF has led to a substantial decrease in the evaluation of seizure behaviors. In line with our findings, some studies declared that growth factors such as nerve growth factors contribute to the attenuation of the seizure in experimental epilepsy by targeting some pathways related to apoptosis (Lei et al., 2017).

In adult mice, the unilateral injection of kainic acid into the hippocampus leads to impairment in the formation and maintenance of memory (Van Den Herrewegen et al., 2019). The disruption of cognitive processes in epileptic rats is associated with neurological disorders in brain structures, including hippocampal regions, astrocytes hypertrophy, and new communication sprouting (Holmes, 2015). In our study, preservation and reminding stored data (as determined and evaluated by STL) were severely disrupted in kainic acid receiving rats. These results support previous studies using the same method for assessing spatial learning (Baluchnejadmojarad & Roghani, 2013) or other evaluation methods (Miltiadous, Stamatakis, Koutsoudaki, Tiniakos, & Stylianopoulou, 2011).

Using HGF significantly improved memory and learning in the present study, both based on Y-maze and passive avoidance learning tests. Kato's previous studies revealed the beneficial effects of HGF on memory and learning regulation under physiological conditions based

on Y-maze and MWM results, which entirely agrees with our results (Kato et al., 2012). Previously, Date et al. also revealed that using hrHGF (HGF) in animals with cerebrovascular disease could improve memory and learning disorders (Date et al., 2004). It has been reported that improvement in memory and learning following HGF administration can be linked to its anti-apoptotic effects (Konishi et al., 1991).

5. Conclusion

Collectively, the present study showed that intra-hippocampal injection of kainic acid could cause tissue and behavioral disorders similar to those found in human TLE. Administering intra-hippocampal HGF can reduce the risk of these complications. Our findings suggested that HGF exerts anti-epileptic effects by targeting astrogliosis and inhibiting apoptotic cell death. All conclusions obtained were compared with the data related to the valproic acid group (as a positive control group), and no significant difference was observed among them.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles are considered in this article. The participants were informed of the purpose of the research and its implementation stages. They were also assured about the confidentiality of their information and were free to leave the study whenever they wished, and if desired, the research results would be available to them. Written consent has been obtained from the subjects. principles of the Helsinki Convention were also observed.

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Authors' contributions

Conceptualization and supervision: Nida Jamali-Raeufy; Methodology: Motahareh Zeinivand and Soraya Mehrabi; Investigation, writing – original draft, and writing – review & editing: All authors; Data collection: Sobhan haghani.

Conflict of interest

The authors declared no conflicts of interest.

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