Neuropeptides 76 (2019) 101932

Contents lists available at ScienceDirect

Neuropeptides

journal homepage: www.elsevier.com/locate/npep

2-Deoxyglucose protects hippocampal neurons against kainate-induced temporal lobe epilepsy by modulating monocyte-derived macrophages (mo-MΦ) and progranulin production in the hippocampus

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ARTICLE INFO

Keywords:
Status epilepticus
Monocyte-derived macrophages
2 Deoxyglucose
Progranulin

ABSTRACT

Inflammation is an important factor in the pathology of epilepsy with the hallmarks of resident microglia activation and infiltration of circulating monocytes in the damaged area. In the case of recovery and tissue repair, some monocytes change to macrophages (mo-MΦ) to enhance tissue repair. 2-deoxyglucose (2DG) is an analog of glucose capable of protecting the brain, and progranulin is a neurotrophic factor produced mainly by microglia and has an inflammation modulator effect. This study attempted to evaluate if one of the neuroprotective mechanisms of 2-DG is comprised of increasing monocyte-derived macrophages (mo-MΦ) and progranulin production.

Status epilepticus (SE) was induced by i.c.v. injection of kainic acid (KA).2DG (125/mg/kg/day) was administered intraperitoneally. Four days later, animals were sacrificed. Their brain sections were then stained with Cresyl violet and Fluoro-Jade B to count the number of necrotic and degenerating neurons in CA3 and Hilus of dentate gyrus of the hippocampus. Lastly, immunohistochemistry was used to detect CD11b + monocyte, macrophage cells, and Progranulin level was evaluated by Western blotting.

The histological analysis showed that 2DG can reduce the number of necrotic and degenerating neurons in CA3 and Hilar areas. Following KA administration, a great number of $cD11b^{+}$ cells with monocyte morphology were observed in the hippocampus. 2DG not only reduced $cD11b^{+}$ monocyte cells but was able to convert them to cells with the morphology of macrophages (mo-MΦ). 2DG also caused a significant increase in progranulin level in the hippocampus. Because macrophages and microglia are the most important sources of progranulin, it appears that 2DG caused the derivation of monocytes to macrophages and these cells produced progranulin with a subsequent anti-inflammation effect. In summary, it was concluded that 2DG is neuroprotective and probably one of its neuroprotective mechanisms is by modulating monocyte-derived macrophages by progranulin production.

1. Introduction

Epilepsy is one of the most common neurological disorders in the world (Vezzani et al., 2016). Although a variety of antiepileptic drugs (AEDs) now exist, one third of epilepsy patients still have persisting seizures which do not respond to drugs (Perucca et al., 2007). Thus, there is an urgent need to develop effective therapies.

2-deoxyglucose (2DG) is a glucose analog which partially inhibits glycolysis and mimics the function of ketogenic diet by the production of ketone bodies (Yao et al., 2011). 2-deoxyglucose exerts acute anti-seizure and chronic anti-epileptogenic actions in animal models (Stafstrom et al., 2008; Stafstrom et al., 2009; Garriga-Canut et al., 2006). There is some evidence indicating the neuroprotective role of 2DG in brain trauma (Hutchinson et al., 2014) and brain ischemia, which show it protects brain cells by increasing ER chaperon GRP78 (Wu et al., 2014; Wei et al., 2003). The underlying mechanism of 2DG is mainly through suppression of glycolysis. However, in culture, 2DG exerts its protection against neurodegeneration by eliminating microglia (Vilalta and Brown, 2014b). Although the cardiotoxicity of 2DG has been reported after chronic use (Minor et al., 2010), this toxicity depends on the concentration. At doses normally associated with anti-epileptic effects it is well tolerated by the body (Singh et al., 2015;

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https://doi.org/10.1016/j.npep.2019.05.003
Received 10 December 2018; Received in revised form 3 May 2019; Accepted 7 May 2019
Available online 08 May 2019
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Ockuly et al., 2012). Surprisingly, unlike most anti-seizure medications, it acts on a broad spectrum and can prevent seizure caused by a variety of mechanisms, including brain-derived neurotrophic factor and its receptor TrKB (He et al., 2004).

Several studies have shown the role of inflammation in the pathogenesis of epilepsy (Zattoni et al., 2011; Turrin and Rivest, 2004; Schultzberg et al., 2007; Marchi et al., 2009). Inflammation is also important in drug-resistant epilepsy. Apart from the two main mechanisms of drug-resistance, alterations in drug transporters and drug targets, some studies emphasize the involvement of brain inflammation and glial changes (Margineanu and Klitgaard, 2009).

Epilepsy induces activation of microglia (resident macrophages in the brain) and activated microglia can produce pro-inflammatory cytokines which are toxic to neurons (Block et al., 2007; Vezzani and Granata, 2005). It has recently been shown that the addition of 2DG in culture induced microglia necrosis while it protected neurons from neurodegeneration (Vilalta and Brown, 2014a). Additionally, activated microglia are not the only cells which mediate brain inflammation during status epilepticus. It has become evident that monocytes and neutrophils also infiltrate the epileptic brain (Libbey et al., 2011; Vinet et al., 2016), although the exact role of these cells is yet to be fully understood. Moreover, progranulin is a neuronal growth factor and modulator of inflammation. Status epilepticus induces expression of progranulin, which potentiates the activation of microglia/ macrophages (Zhu et al., 2013).

To the authors' knowledge, no existing research has addressed possible relationship between 2DG, progranulin and activated microglia/macrophages *in vivo*. In view of the important role of inflammation in epilepsy, targeting brain inflammation is a potential therapy for drug-resistant epilepsy.

This study attempted to show that 2DG may exert its neuroprotective effects by modulating macrophage infiltration and progranulin expression.

2. Materials and methods

2.1. Materials

Kainic acid and 2DG were purchased from Sigma Chemical Co. (St. Louis, MO, USA) Antibodies were purchased from Biorbyt Co. (England).

2.2. Animals

Forty male Wistar rats (200–250 g) were randomly divided into five groups: the control vehicle group (n = 8), Sham operated group (n = 8), 2DG alone group (n = 8), KA group (n = 8) and KA + 2DG group (n = 8). *Data* from sham, 2DG alone and control groups were *pooled* together, *as there was no significant difference between* them.

Rats were housed in individual cages under controlled conditions (22 ± 2 °C, 12/12 h light/dark cycle, lights on at 8.00 a.m.) with water and food *ad libitum*. Experiments were performed in accordance with the rules of the Institutional Animal Care and Use Committee of the [Tehran University of Medical Sciences (TUMS)] (IACUC protocol number: [93-02-45-26666]).

2.3. Experimental design

From the 40 rats, 16 underwent Kainate-induced status epilepticus. SE was defined as continuous limbic motor seizure activity (facial and forelimb clonus, head bobbing, and facial twitching) uninterrupted by normal behavior such as grooming (Kondratyev and Gale, 2004). Eight controls received saline injections instead of KA. Kainic acid (KA, Sigma-Aldrich) was dissolved in saline and injected with a final concentration of 0.8 μg/μl. Among the 16 rats that underwent SE, 8 were treated for 4 days with 2DG which was administered intraperitoneally (125 mg/kg, Sigma U.S.A.), once daily, starting at 30 min before KA microinjection. To determine the optimal dosage of 2DG, three doses of 2DG (125, 250 and 500 mg/kg) were used in the pilot study. Our preliminary data showed that 2DG at 125 mg/kg was more effective. We, therefore, applied 125 mg/kg for all experiments.

The seizure was induced by KA injection into the left lateral cerebral ventricle. For this purpose, rats were first anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) *i.p.*, and then immobilized using a stereotaxic apparatus. After exposing the skull, a burr hole was made using an electrical microdrill according to the following coordinates: 1 mm posterior to the bregma, 1.5 mm lateral to the midline, and 3.5 mm under the dura (Paxinos and Watson, 2006). In each rat, KA (0.8 μg in 1.0 μl saline) was injected at a speed of 0.2 μl/min. The KA-treated rats were behaviorally monitored for 3 h *after* recovery from *anesthesia*. Seizures were rated according to Racine's scale (Racine, 1972). Seizure assessment and histological analysis was performed by an experimenter blind to animal treatment.

2.4. Fluoro- Jade B cell death staining

Rats were sacrificed for histological analysis of neuronal death by FJB (Histo-Chem, USA) 4 days after KA-induced seizure. Rats were deeply anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg, *ip*), and transcardially perfused with saline, followed

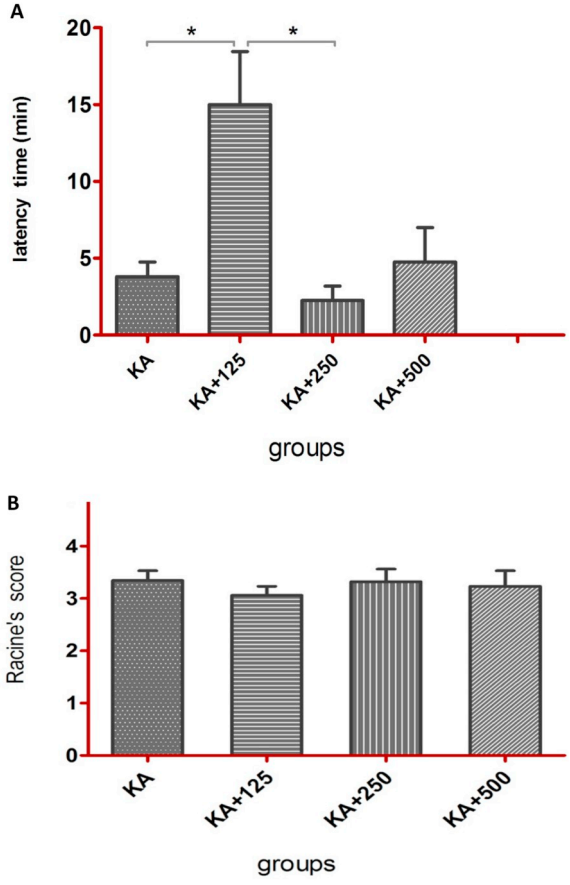

Fig. 1. 2DG at the dosage of 125 mg/kg increases latency to seizure (A). However, 2DG is not effective to reduce severity of seizure (B).

by 4% paraformaldehyde dissolved in PBS. The brains were removed immediately and postfixed with 4% paraformaldehyde overnight at 4 °C then the brains were rehydrated and embedded in paraffin. After microtome sectioning, 10 µm tissue sections were mounted on gelatinized slides. Six sections from each brain at the same level of hippocampus were stained for FJB as previously described (Yeganeh et al., 2013). Briefly, sections were first immersed in Xylene for 10 min two times, absolute ethanol for 5 min two times, and then sequentially in 90% ethanol 2 min and 1% sodium hydroxide in 80% ethanol, and distilled water for 2 min. Thereafter, the slices were transferred to 0.06% potassium permanganate solution for 15 min, rinsed gently, and stained with 0.0004% FJB and 0.1% acetate for 30 min in the dark. The degree

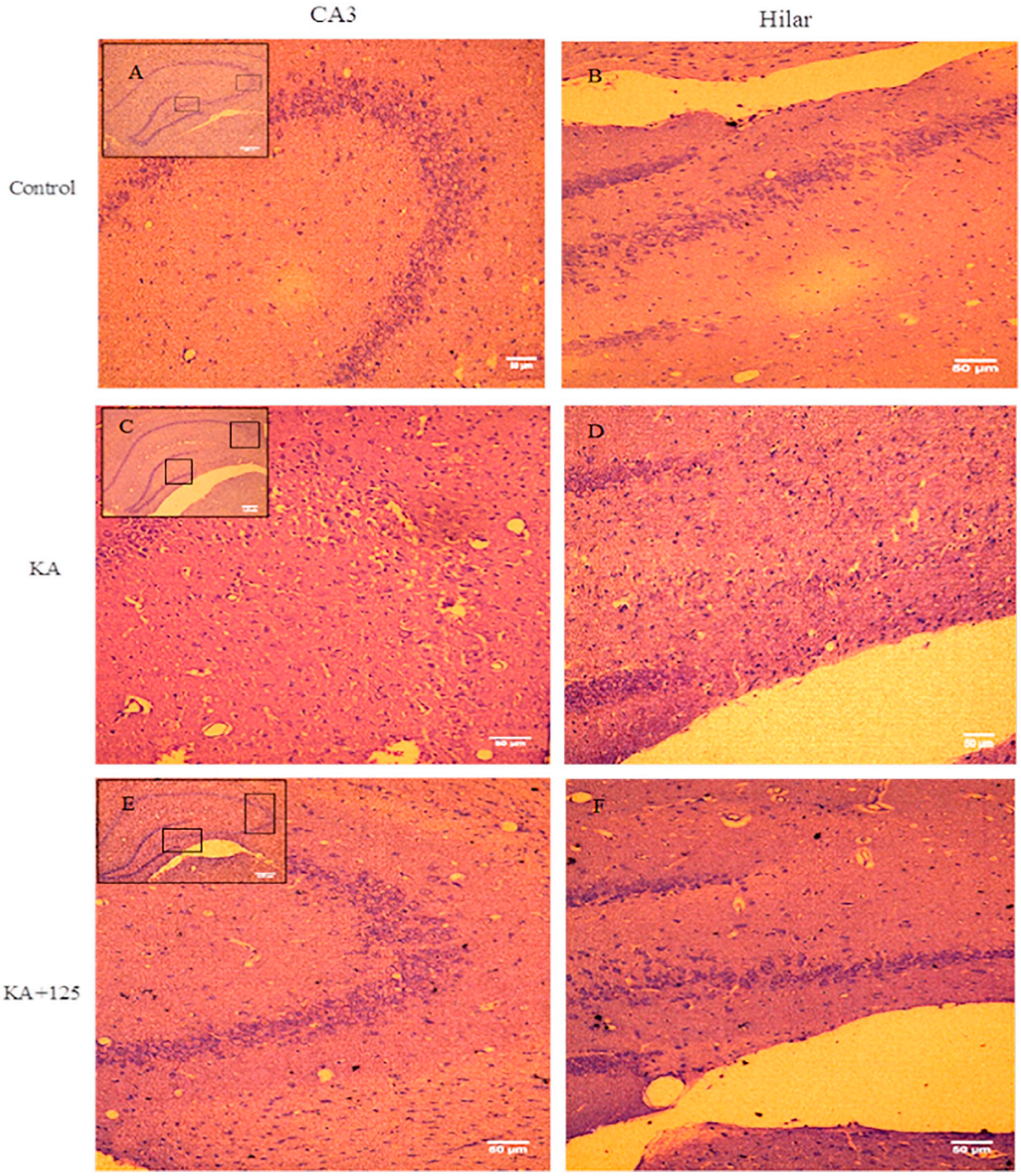

Fig. 2. 2DG protects hippocampal CA3 and hilar neurons against KA neurotoxicity. Viable neurons were counted and analyzed at 96 h after KA injection. Viable neurons were significantly decreased in the CA3 and hilar regions in KA groups compared with those in the control groups. In CA3 area, the architecture of neurons was disrupted in KA groups and restored following 2DG treatment. Viable neurons were significantly increased in KA + 125 groups compared with those in KA groups. Scale bar: 50 µm.

of neuronal death was assessed by counting FJB positive neurons in the hippocampal CA3 pyramidal layer and hilus of the dentate gyrus under an epifluorescent microscope with excitation light (450–490 nm).

2.5. Nissl staining

Six sections from each brain were de-waxed and rehydrated, stained with 0.1% cresyl violet solution (dissolved in 0.01% glacial acetic acid) at 37 °C for 10 min, and checked microscopically. Surviving neurons were quantified at 400× magnification. The number of intact cells was counted in the CA3 and hilar regions and the average number of cells was calculated for analysis.

2.6. Immunohistochemical analysis

Paraffin sections were de-waxed and rehydrated in the same manner as the Nissl staining. Briefly, endogenous peroxidase was blocked by 3% hydrogen peroxide. Antigens were unmasked in a water bath at 70 °C for 30 min. Incubation was performed using normal goat serum for 30 min. Incubation with rabbit anti-rat OX-42 monoclonal antibody (1:70; BIORBYT, England) was done in a wet chamber at 4 °C overnight. Incubation with goat anti-rabbit HRP secondary antibodies (1:100 Biorbyt, England) was performed at room temperature in a wet chamber for 30 min. Staining with 3,3′-diaminobenzidine was done (Sigma Co., USA), and the total number of positive cells were counted in an area of about 875 μm ^{2} between the CA1 subfield and the Dentate Gyrus.

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2.7. Western blot analysis

Measurements of the progranulin to β-actin ratio were determined by western blotting. The hippocampus was isolated and homogenized in a cold lysis buffer (Ripa 1 ml and inhibitor cocktail 10 μl Protein) followed by centrifugation at 13,000g, 5 min at 4 °C. The supernatant was used for measurement of proteins. Protein concentration was determined using the BIO -RAD Kit USA. Protein samples (40 μg per lane) were separated on a 12% SDS-polyacrylamide gel and then transferred onto polyvinylidene fluoride (PVDF) membranes for immunoblotting. The membranes were blocked with Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST) and 5% fat-free milk for 1 h at RT. The membranes were then incubated (overnight at 4 °C) with rabbit antibodies against progranulin (1:500 Biorbyt), and β-actin (1:1000 Biorbyt), followed by 1 h of RT incubation with a peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG, 1:700 Biorbyt). All Western blotting data were representative of at least three independent experiments. The relative intensities of progranulin were normalized to the internal reference protein β-actin and then normalized to the control group. Image J software was used for densitometric analysis.

2.8. Statistical analysis

For multiple comparisons, one- way ANOVA or Kruskal–Wallis, (for non-parametric data) and Scheffe's *post hoc* test was performed. All data were expressed as mean (± SEM). P values < .05 were considered significant. All statistical were performed using SPSS version 16 and GraphPad Prism 5.0 software.

3. Results

3.1. Behavioral results

There was no significant difference between the KA and KA + 125 groups for seizure intensities, this could imply that the 2DG had no anticonvulsive effect at least at this dosage. However, a significant difference in the latency to the onset of seizure activity was shown after treatment with 2DG 125 mg/kg. Other dosages of 2DG were not effective (Fig. 1A&B).

Data are mean ± SEM (n = 8 rats). One-way ANOVA followed by post- hoc tests revealed significant differences between KA and KA + 125 (P < 0.05). *P < 0.05 compared with KA & KA + 250.

3.2. Treatment with 2DG decreases cell loss in hippocampus

Four days after KA injection, extensive cell loss was observed in the CA3 and hilar areas in Cresyl violet and FJB staining. In Cresyl- violet stained sections, the CA3 layer almost disappeared. Figs. 2 and 3 shows that treatment with 2DG not only significantly protects neurons, but also preserves CA3 layer architecture.

Consistent with the Nissl staining results of neuronal death found in the kainate model, Fluoro-Jade B staining showed extensive neuronal death in all KA-treated rats following kainate status epilepticus in neurons from the dentate hilus and CA3 regions (Fig. 4A). In contrast, less Fluoro-Jade B positive neurons were observed in rats treated with 2DG following status epilepticus. Fluoro-Jade B was never observed in control rats that did not receive kainate.

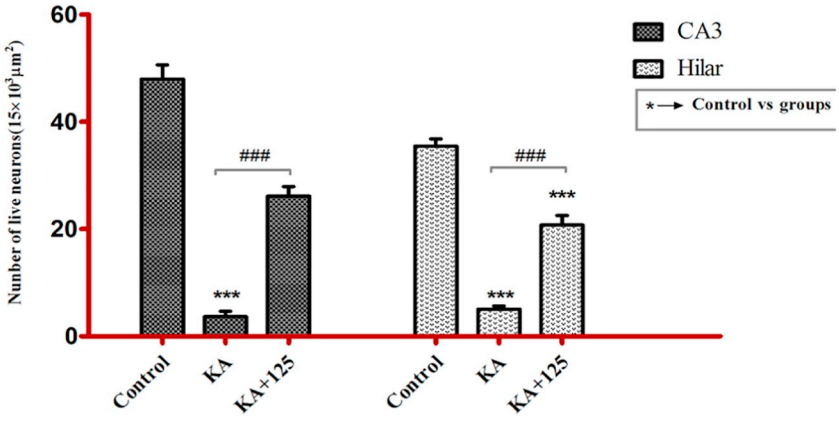

Fig. 3. The effect of 2DG on numbers of intact neurons in the hippocampus (CA3 and hilar areas) in rats 4 days after KA administration by Cresyl violet staining. Data are mean ± SEM (n = 8 rats). One-way ANOVA revealed significant differences between KA and KA + 2DG (P < 0.001). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.) ***P < 0.001 compared with control/vehicle group. ###P < 0.001 compared with KA group control group, KA = kainic acid-treated group, KA + 125 = kainic acid-treated + 2-DG group.

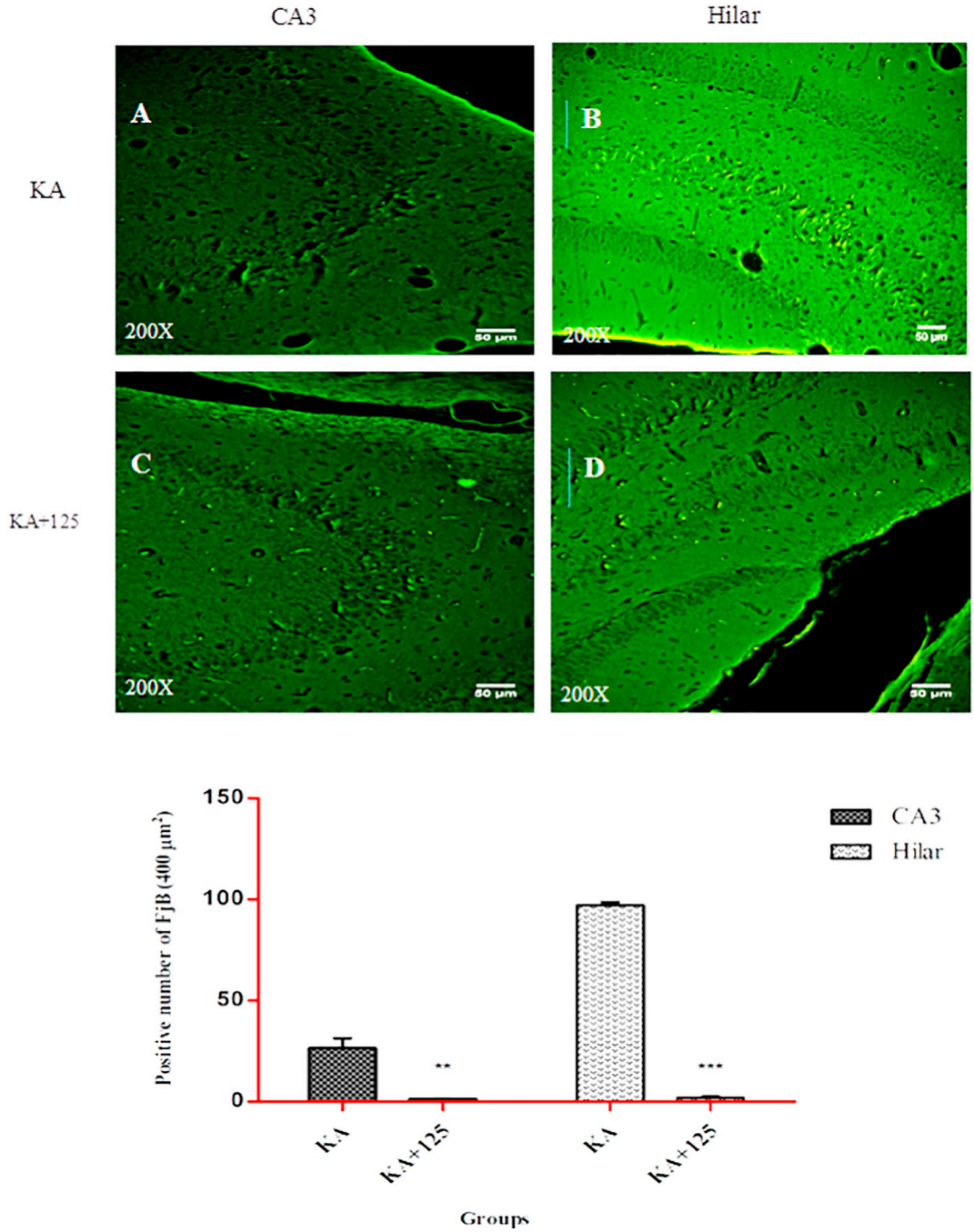

Fig. 4. photomicrographs of Fluoro-Jade B staining show degenerating neurons in CA3 and hilar areas (A, B) respectively of KA groups and (C, D) of KA + 125 groups. The number of Fluoro-Jade-positive cells was reduced in KA + 125 group. Quantitative analysis of Fluoro-Jade B positive neurons in CA3 and hilar areas of the hippocampus demonstrated abundant Fluoro-Jade B positive neurons in KA group and a significant decrease in 2DG treated group (KA + 125). Mean ± SEM. $^{**}P < .01$ and $^{***}P < .001$.

3.3. 2DG alters morphology of CD11b+ cells in the hippocampus

Immunohistochemistry staining was tested for CD11b as a marker of monocyte/ macrophages. The results revealed an abundant number of CD11b + cells in the dorsal part of the hippocampus in KA group with monocyte morphology Fig. 5. In 2DG treated group the number of monocyte CD11b + cells decreased and extensive cells with the morphology of macrophages appeared.

3.4. 2DG increases the level of progranulin in the hippocampus

The presence of progranulin was revealed by the Western blot analysis of all groups. After KA injection, the level of progranulin did not increase significantly compared to control group. However, treatment with 2DG increased the level of progranulin Fig. 6.

After treatment with 2 deoxyglucose, progranulin increased significantly. The expression of β-actin was evaluated as a loading control.

All western blot analyses were run in triplicate to ensure that results were consistent.

4. Discussion

The histological analysis revealed the protective effect of 2DG (125 mg/kg) against KA-induced status epilepticus in rat. 2DG was also effective in retarding the onset of seizure activity, although it could not attenuate seizure severity. An earlier study has shown a positive correlation between neurodegeneration and delay in seizure onset (Kondratyev and Gale, 2004).

It has been previously shown that 2DG can exert neuroprotective effect in the brains of animals subjected to a variety of insults, including epileptic seizures (Garriga-Canut et al., 2006). However, some studies have shown a pro convulsive property for 2DG (Gasior et al., 2010). It appears that 2DG is working strictly in a dose-dependent manner; while in some doses it is protective due to inhibiting glycolysis, in other doses it is harmful by reducing the glucose level in the cells. Consistent with the protective data, the results of this study have shown a strong neuroprotection against kainate at a dosage of 125 mg/kg.

The exact mechanism by which 2DG protects the brain in epilepsy is unclear; however, modulation of brain inflammation during the seizure is probably one underlying mechanism. A recent study has shown that addition of deoxyglucose induced microglial loss and prevented spontaneous neuronal loss in co-culture of neurons and glia (Vilalta and Brown, 2014a).

Microglial cells, the resident macrophages of the brain (Kettenmann et al., 2011), can be activated by inflammation or pathological changes in the brain and can become toxic and/or protective to nearby neurons (Brown and Neher, 2010; Sierra et al., 2015). For many years it has been believed that microglia are the only cells that mediate brain inflammation. However, it has been recently demonstrated that neutrophil and monocytes infiltration damaged the brain and contributed to brain inflammation especially after status epilepticus (Ji et al., 2007; Varvel et al., 2016).

In the results obtained in this study, extensive round CD11b positive cells in the hippocampus were observed 4 days after KA injection. CD11b belongs to the α subunits of the integrin receptor family and is a leukocyte-specific receptor which is used as a marker for monocyte/macrophages and granulocytes (Fagerholm et al., 2006). These cells were considered as infiltrated monocytes in the KA group, although they could be easily mistaken as neutrophils as both are small, round and cd11b positive cells. The hallmark of neutrophils is the morphology of their nuclei unlike monocytes, neutrophils are polymorphonuclear. The other hallmark is the time of appearance in an injured brain. In contrast to monocytes which remain in the injured brain for several weeks (Jeong et al., 2013), neutrophils are found 1–3 days after injury (Fleming et al., 2006). Therefore, it can be concluded that the CD11b positive cells in the hippocampus in our kainate group are infiltrated monocytes.

Interestingly, following 2DG treatment most of the infiltrated monocytes disappeared and were replaced by big CD11b positive cells with morphology of macrophages. These cells were considered as monocyte-derived macrophages (mo- MΦ). The crucial role of these monocyte-derived macrophages in brain repair has been previously shown in a study (Shechter et al., 2009). These cells have the ability to produce IL-10, an important immunosuppressive cytokine (Staples et al., 2007).

It is important to note that, infiltrating monocytes cause brain inflammation and their abundance after status epilepticus in KA group exacerbates neuronal damage (Varvel et al., 2016); however, in the case of recovery monocytes changed to macrophages which are vital for brain recovery.

It appears that 2DG is able to trigger the production of monocytes-derived macrophages in epileptic brains.

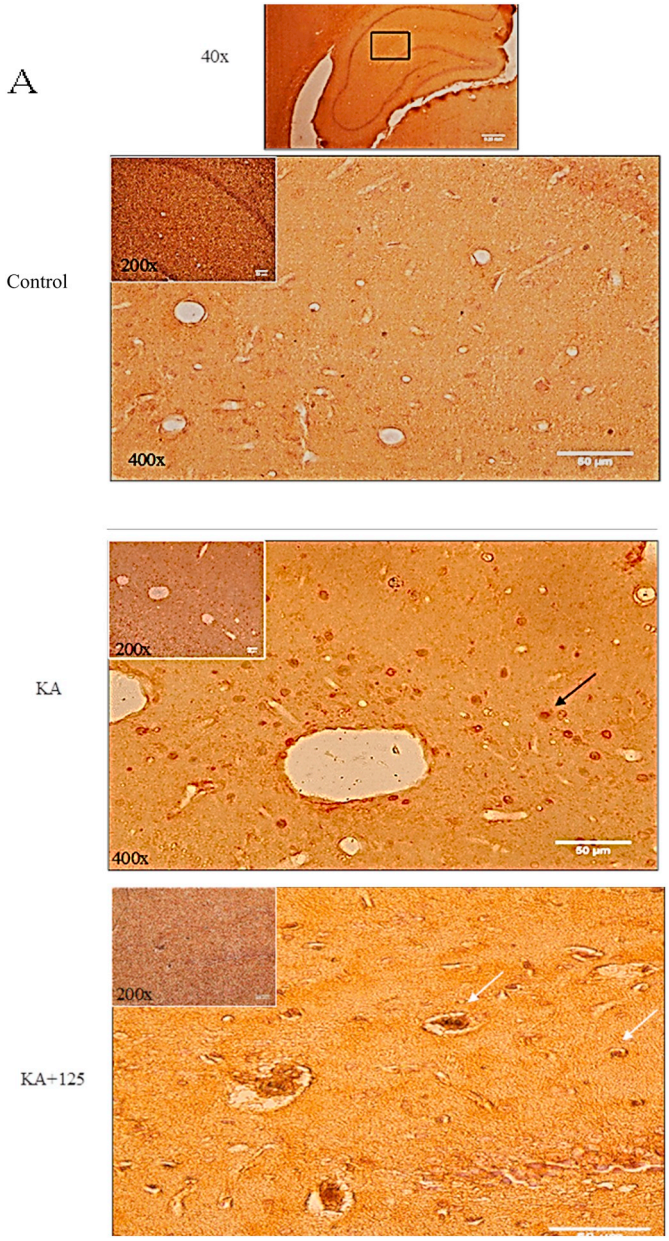

Fig. 5. A: An immunohistochemistry staining of CD11b positive cells in control, KA and KA + 125 groups. After KA injection, abundant CD11b positive cells with the morphology of monocyte were stained in the hippocampus. Treatment with 2DG converted these cells to CD11b positive cells with the morphology of macrophages. White arrows represents macrophages and black arrows represents monocytes. B: Quantification of positive immunohistochemical staining. Graphs showing quantification of immunopositive cells for monocytes CD11b + positive cells upper, and macrophages CD11b on the lower. * shows significance compared to control group and # compared to KA group.

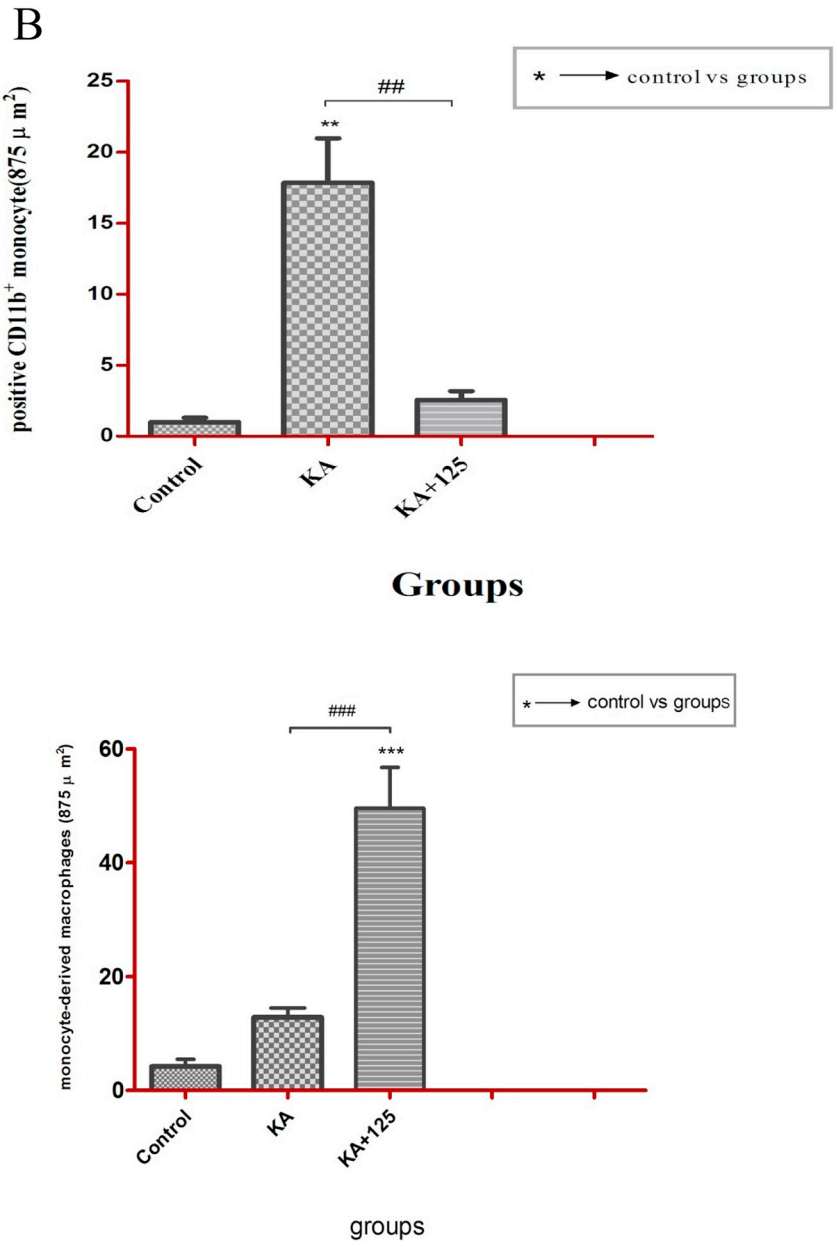

Fig. 5. (*continued*)

The results also revealed that 2DG increased the progranulin level in the brain. Several studies have been conducted regarding the protective effect of this neurotrophic factor (Jackman et al., 2013; Gass et al., 2012). Progranulin, as well as many other neurotrophic factors, are important in modulating epilepsy (Kovac and Walker, 2013; Iughetti et al., 2018).

Interestingly, it has been well established that progranulin is involved in the recruitment of immune cells in the brain (Pickford et al., 2011). Progranulin can suppress neuroinflammation by inducing production of IL-10 in microglia (Kanazawa et al., 2015). In addition, it has been demonstrated that after stroke M2 like or anti-inflammatory monocyte/macrophages induced secretion of growth factors like BDNF, progranulin and IL-10, which could promote tissue recovery (Kanazawa et al., 2017). Progranulin is the key factor in monocyte-derived macrophages activation and brain recovery following the application of 2-Deoxyglucose.

Based on the authors' knowledge, this is the first study that addresses any relationship between 2DG, MΦ cells, and progranulin.

Further works are needed to reveal the exact role of progranulin and MΦ cells and the source of IL10 production cells in 2DG neuroprotective effect.

Overall, we concluded that 2DG is neuroprotective, and in all probability one of its neuroprotective mechanisms is by derivation of monocytes to macrophages which *per se* may produce anti-inflammatory markers.

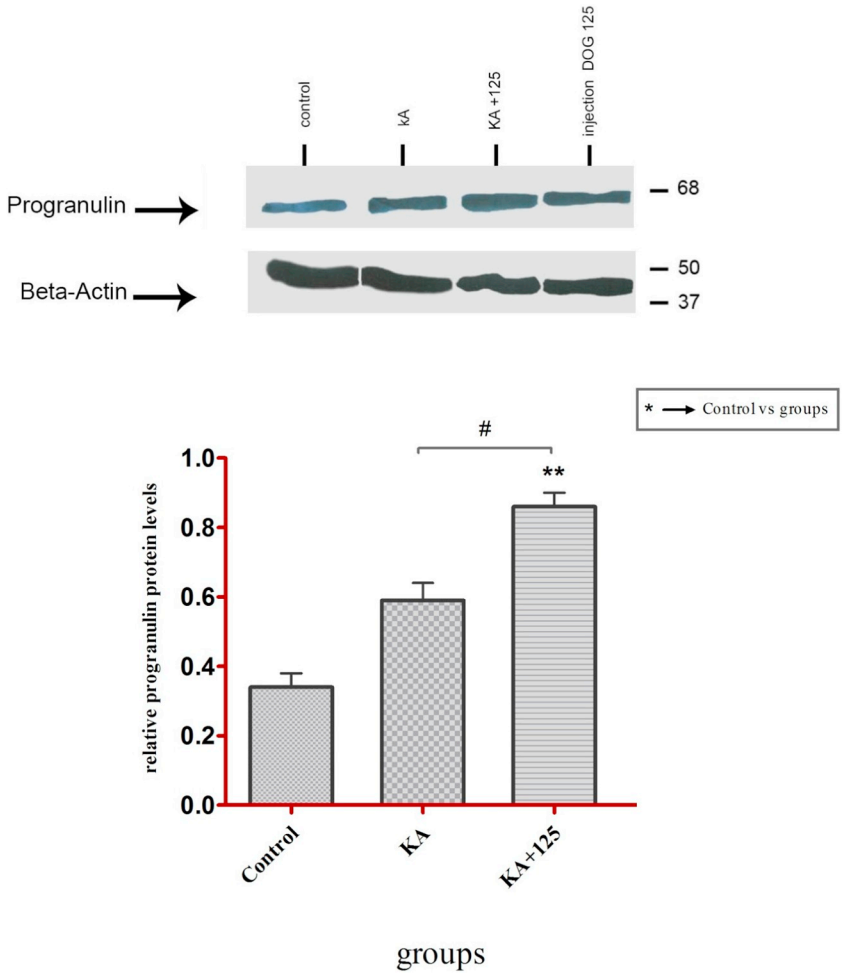

Fig. 6. Results of Western blot analyses of progranulin in 3 different groups. Analyses of progranulin in control and KA group revealed no significant increase.

Declaration of competing interests

All authors declare that there is no conflict of interest.

Acknowledgments

This research has been supported by a grant (No 25387) sponsored by Iran University of Medical Sciences, Iran.

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