Original Article
Effect of intra-cisternal application of kainic acid on the spinal cord and locomotor activity in rats

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Received May 18, 2013; Accepted June 27, 2013; Epub July 15, 2013; Published August 1, 2013

Abstract: Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease of idiopathic etiology. Glutamate excitotoxicity is one of the proposed hypotheses causing progressive death of motor neurons. We aimed to develop an experimental animal model of this disease to enhance the knowledge of pathophysiological mechanism of ALS. Male Wistar rats were infused with Kainic acid (KA) intra-cisternally for 5 days at the dosage of 50 fmol/day and 150 fmol/day. Locomotor activity, sensory function and histological changes in cervical and lumbar sections of spinal cord were evaluated. Glial Fibrillary Acidic Protein (GFAP) and Neurofilament Protein (NFP) were used as immunohistochemical marker for reactive astrogliosis and neuronal damage respectively. Specific Superoxide Dismutase (SOD) activity of spinal cord was estimated. The locomotor activity in the parameter of observed mean action time remained reduced on 14th day after administration of KA. Spinal motor neurons under Nissl stain showed pyknosis of nucleus and vacuolation of neuropil. GFAP expression increased significantly in the lumbar section of the spinal cord with high dose of KA treatment (p<0.05). NFP was expressed in axonal fibres around the neurons in KA-treated rats. A significant increase in specific SOD activity in both cervical and lumbar sections of the spinal cord was found with low dose of KA treatment (p<0.05). This study concludes that spinal cord damage with some features similar to ALS can be produced by low dose intra-cisternal administration of KA.

Keywords: Amyotrophic lateral sclerosis, kainic acid, glial fibrillary acidic protein, neurofilament protein, superoxide dismutase, spinal cord

Introduction

Kainic acid (KA), an agonist for ionotropic glutamate receptors, is a natural seaweed toxin which induces neuropathological changes both in vivo and in vitro and is commonly used to study the mechanism of excitation-induced neuronal apoptosis [1]. The molecular formula of KA is C_{10}H_{15}NO_{4} with a molar mass of 213.23 g/mol. LD_{50} of KA in rodents is 100 μM in presence of magnesium ions (Mg^{2+}) [2]. Stimulation of glutamate receptors by KA causes intra-cellular calcium ion influx, leading to production of free radicals and ATPase which set off further influx of harmful ions and chemicals causing neuronal death [3]. This mechanism was related to various neurological disorders like Amyotrophic Lateral Sclerosis, Alzheimer’s disease as well as Parkinson's disease [4]. Similar mechanism was also explained for the status epilepticus induced by KA. Systemic administration of KA induces limbic motor seizures [5]. The hippocampal region was most susceptible to neuronal injury by systemic administration due to the presence of dense KA receptors in the region [6]. However sub-convulsive dose of KA was found to produce changes in the spontaneous behavior [7] and long-term hyperalgesia [8]. Up-regulation of Glial fibrillary acidic protein (GFAP) was found to play a key role in the survival of pyramidal neurons and GFAP was used as a biological marker of neurotoxicity after cerebral injury [9].
the selective death of both the upper and lower motor neurons which ultimately would lead to progressive paralysis [10]. Loss of motor neuron was the principal finding bridging the disease pathophysiology and clinical expression. Approximately two-thirds of the ALS patients had a spinal form of the disease which started on a limb with focal muscle weakness. ALS was divided into two categories; sporadic ALS (SALS) and familial ALS (FALS). Sporadic form was found in approximately 95% of all ALS cases while FALS was found in the remaining 5% [3]. The hypotheses proposed as the etiology of SALS include production of autoantibodies to calcium channels, neurofilament accumulation [11], mitochondrial dysfunction and excitotoxic death arising from mishandling of glutamate [10].

A review of existing literature shows that most of the existing models of ALS are based on animal models carrying transgene for mutant human SOD [12-14]. These models reflect the pathophysiology of the FALS. Experimental animal model with pathophysiological basis of SALS in adult animal has not been reported. This study was planned to evaluate the neurological and histological changes in the spinal cord towards producing an experimental ALS model in rat via intra-cisternal administration of KA. An experimental model of ALS would offer a better understanding of the disease progression and facilitate further interventional strategies.

Material & methods

Kainic acid

KA or 2-Carboxy-3-carboxymethyl-4-isopropenylpyrrolidine was supplied by Sigma-Aldrich, Malaysia. An amount of 10 mg/mL of KA stock in sterile normal saline was used to prepare 50 fmol/mL and 150 fmol/mL (0.000005% and 0.000015% of LD50 of KA) of sterile KA solution under aseptic precaution. Two KA dosage in low concentration, 50 fmol/day and 150 fmol/day were used in the experiment. A pilot study was conducted to estimate the dosage which would not produce seizures. The intra-cisternal administration could directly mix with the cerebrospinal fluid to produce an immediate effect [15].

Experimental animals

Twenty-four male adult Wistar rats (10 weeks old) (weighing 280 g to 330 g) were obtained from the Specific Pathogen Free (SPF) Facility in Brain Research Institute, Monash University Sunway Campus, Malaysia. They were housed in autoclaved plastic cages and maintained in a 12-hour light-dark cycle with access to rat-chow and water ad libitum. The rats were acclimatized to the environment 7 days prior to the experiment. They were then randomly divided into three groups (n=8): control (C) treated with normal saline, K1 group treated with 50 fmol/day of KA and K2 group treated with 150 fmol/day of KA. All procedures and the handling of animals were performed according to the standard protocol of the International Medical University Research and Ethics Committee and Monash University, Sunway campus, Malaysia, Animal Ethics Office. The Animal Care Ethics approval was obtained as per standard procedure.

Experimental paradigm

The timeline for all procedures is presented in Figure 1. All rats underwent baseline neurological test at Day 1 of the study and from Day 2 until Day 6 they received intra-cisternal injections of KA. Three subsequent neurological tests were conducted on Day 8, Day 14 and Day 20 (48 hours, 8th day and 14th day after the completion of KA/saline treatment) respectively. On Day 22, the animals were sacrificed under ether anaesthesia to collect spinal cord specimens which were used for histological and biochemical studies.

Treatment by intra-cisternal injections

Two groups (K1 and K2) received sterile KA solution and the control (C) group received sterile normal saline solution. Each intra-cisternal injection was administered with 10 µL of sterile solution. Group C received normal saline solution, Group K1 received 50 fmol/day of KA solution and Group K2 received 150 fmol/day of KA solution. The intra-cisternal injections were administered for five consecutive days. All animals were first anaesthetized with intraperitoneal injection of Ketamine/Xylazine (0.25 mL per 100 grams). Toe-pin test was done to check the consciousness level (to achieve sur-
gical anaesthesia). When there was no reflex present, the rats were mounted on the stereotaxic instrument and the intra-cisternal injections were administered with aseptic precautions. Reflux of clear CSF into the syringe confirmed the administration [16].

Locomotor activity and sensory function

Tests for locomotor activity and sensory function were done one day prior to injections and three times after the experiment (48 hours, 8 days and 14 days after the last intra-cisternal injection). Open Field Test (OFT) was done to evaluate changes in the locomotor activity of the rats. The animal was placed at one corner of a 120 x 120 cm black box and the locomotor activity was recorded with a video camera for 15 minutes [17]. The tests were done under red light as the rats were blind to the red light. The analysis of the action time was done using the LoliTrack 2.0 software (Loligo System, Denmark). Grooming action was taken into account as forelimbs were used when the animal groomed itself. Hot Plate Test (HPT) was done to evaluate the changes in the sensory nociception of the rats. A standard temperature was determined by placing a control rat on the hot plate with the temperature being raised slowly and stopped when the rat started licking its hind limb paws. The mean temperature calculated for all the control rats was used as the standard temperature (55°C). The latency time for the sensation of temperature (licking by the rats of either hind limb paw) was noted and compared group wise. An increase in latency time indicated loss of sensory nociception with increased threshold for response to increased temperature.

Preparation of tissue for analysis

The rats were sacrificed under ether anaesthesia on the sixteenth day following the last intra-cisternal injection. Five rats from each group were given intra-cardiac perfusion of 4% Paraformaldehyde and the spinal cord samples from the cervical and lumbar regions were collected for histological analysis. Rest of the three rats from the groups did not receive intra-cardiac perfusion and the spinal cord samples were stored in phosphate buffer saline (PBS) at -80°C [18]. These spinal cord samples were homogenized and used for superoxide dismutase (SOD) estimation. During dissection, entire vertebral column was removed and a laminectomy was done to expose the spinal cord. Then the portions of the cervical and lumbar parts of the spinal cord were cut and fixed in 4% Paraformaldehyde for 24 hours. The spinal cord tissue samples were processed and embedded into paraffin blocks. Using a Leica microtome serial sections were done.

Histology and immunohistochemistry

Histological sections of 8 µm obtained from the cervical and lumbar parts of the spinal cord were stained with 0.2% Thionin (Nissl stain). Nissl stain was used to observe the morphological changes in the motor neurons of the spinal cord. The spinal cord sections were deparaffinised in xylene, rehydrated in alcohol of different concentrations, immersed into 0.2% Thionin solution for twenty minutes and finally dehydrated with alcohol followed by clearing with xylene. For both glial fibrillary acidic protein (GFAP) and neurofilament protein (NFP) immunohistochemical study, spinal cord paraffin sections of 4 µm were deparaffinized in
xylene and hydrated through graded alcohol concentrations. The sections were pre-treated with heat-induced-epitope-retrieval (HIER) solution using a digital decloaking chamber (DAKO Envision™ FLEX Target Retrieval Solution). Following a rinse in solution of Tris-buffered saline with Tween 20 (TBST), pH 7.6, the slides were incubated with Peroxidase Blocking Reagent (H₂O₂) for five minutes. For identification of GFAP positive astrocytes, the sections were then incubated with primary polyclonal rabbit antibody (FLEX Polyclonal Rabbit Anti-GFAP, Ready-to-Use, DAKO, Malaysia) for thirty minutes, followed by incubation with Dako REAL Envision HRP polymer for thirty minutes. The ready-to-use antibody was not diluted and application volume was 1 x 200 μL per slide [19]. The slides were then rinsed with TBST before incubating with DAB substrate for ten minutes to develop immuno-staining. Finally, the slides were counterstained with Haematoxylin for two minutes and washed with distilled water before the dehydration process. For identification of neurofilaments in the axonal fibres of the neuron, the spinal cord sections were incubated with monoclonal mouse anti-human neurofilament antibody (FLEX Monoclonal Mouse Anti-Human Neurofilament Protein, Ready-to-Use, Clone 2F11, DAKO, Malaysia) for sixty minutes, followed by incubation with Dako REAL Envision HRP polymer for thirty minutes. The microphotographs of five GFAP-stained spinal cord sections (every 10th section) of cervical and lumbar regions from five animals in each group were used for the count of GFAP positive glial cells using Nikon NIS-Elements software. The number of astrocytes, stained brown with GFAP, within a rectangular area of 150 x 150 μm was counted. The counting was done by an unbiased student blinded with the identity of the sections. Mean count of GFAP positive astrocytes was calculated per square micron of spinal cord section.

**Estimation of SOD**

Spinal cord sections were homogenized using 5-10 mL of cold HEPES buffer per gram of spinal cord tissue and centrifuged at 1500 x g for five minutes at 4°C to obtain the supernatant. Supernatants were stored at -80°C and used to estimate the amount of superoxide dismutase (SOD) concentration in the spinal cord samples. 200 μL of diluted radical detector and 20 μL of diluted xanthine oxidase were added into 10 μL of homogenized spinal cord tissue in a 96-well microplate. The microplate was incubated on a shaker for twenty minutes at room temperature. Absorbance of the SOD standards and spinal cord homogenate were read at 450 nm using a Multifunctional Microplate Reader (Tecan Infinite F200 Series). Using the absorbance readings from the microplate reader, a standard graph was plotted. Using Minitab and Microsoft Excel, the mean fluorescence readings were substituted into the equation of the linear regression line obtained from the positive controls (control sample with known concentration of SOD supplied with the kit). Thus SOD activity of the experimental samples (U/mL of the homogenate) was calculated. A procedure for determining the protein concentration of each spinal cord homogenate sample was carried out using Quick Start™ Bradford dye reagent (Bio-Rad Lab) with bovine serum albumin (Sigma-Aldrich) as a standard. Thus the protein concentration in 1 mL of spinal cord homogenate was calculated [20]. Finally the SOD activity of the spinal cord samples (U/mL) was converted to unit per mg (U/mg) of protein concentration.

**Statistical analysis**

Neurological examination data in the OFT and HPT test for each group were analyzed by repeated-measure ANOVA test (SPSS 17). Mean count of GFAP positive astrocytes per square micron of spinal cord sections and specific SOD activity data (U/mg) were subjected to one-way analysis of variance (ANOVA) statistical analysis followed by the Bonferroni post hoc test (SPSS 17). Probability values (p) of less than 0.05 were considered as significant differences. Data were presented as the means ± SEM.

**Results**

Following initial KA injections, the rats had stippled red pigmentation on their fur, porphyrin discharge from their eyes and nose and vocalization with touch.

**Locomotor activity and sensory function**

In the open field test to observe locomotor activity, following KA administration, a state of hyperactivity was observed 48-hours after the experiment in both the KA-treated groups. Further observation revealed a reduction in
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locomotor activity and forelimb paralysis in rats from both experimental groups compared to the control group. The mean action time was reduced in both group K1 and K2 on the 8th day of the experiment compared to the control group. In group K2 the reduction was persistent until the 14th day (Figure 2). At the end of the 14th day of the experiment, when compared to Group C the overall locomotor activity expressed by mean action time in seconds was reduced in Group K2 (-20.28%) followed by Group K1 (-11.46%) (Figure 2). The reduction in mean action time in group K1 and K2 was not statistically significant.

In the Hot Plate Test, mean latency time for the response to rise in temperature was increased (increased pain threshold) after 48-hours in both KA-treated groups compared to the control group. Subsequently on the 8th and 14th day of the experiment, there is reduction in the mean latency time but the values remained above the pre-experiment level (Figure 3). At the end of the 14th day of the experiment, when compared to Group C the mean latency time was increased in Group K2 (+27.00%) followed by Group K1 (+15.69%) (Figure 3). The increase in mean latency time in group K1 and K2 was not statistically significant.

Histology and immunohistochemistry

Qualitative analysis of the spinal cord sections under Nissl stain showed healthy prominent multipolar neurons with clear nucleus and nucleolus in the ventral gray column of the spinal cord of group C rats (Figure 4, Nissl-C). A few neurons showed loss of nucleolus and pyknosis of nuclei in group K1 (Figure 4, Nissl-K1). Neuronal damage was more clearly visible in group K2 in the form of pyknosis of nuclei, large vacuolation of neuropil around the damaged neurons in both cervical and lumbar sections (Figure 4, Nissl-K2). On observation of the serial sections, neuronal damage was more prominent in the ventral gray horn of the lumbar spinal cord sections compared to the cervical sections.

Figure 2. The changes in the locomotor activity before and during the experiment expressed in bar charts of Mean Action Time in seconds (± S.E) in Open Field Test. Group C- normal saline, Group K1- 50 fmol/day of KA, Group K2- 150 fmol/day of KA.

Figure 3. The changes in the sensory nociception before and during experiment expressed in bar charts of Mean Latency Time in seconds (± S.E) in Hot Plate Test. Group C- normal saline, Group K1- 50 fmol/day of KA, Group K2- 150 fmol/day of KA.
Qualitative analysis of the spinal cord sections under GFAP immunostaining showed a few fibres of the glial cells stained brown in the ventral gray horn of spinal cord sections in the control group (Figure 4). GFAP positive astrocytes with radiating processes stained brown under GFAP immunostaining were observed in spinal cord sections in KA-treated group K1 and K2 (Figure 4, GFAP-K1, GFAP-K2). The brown stained processes of the glial cells were more prominently seen in spinal cord sections of the group treated with low-dose of KA (Figure 4, GFAP-K1). An overall increase in the mean density of GFAP positive astrocytes was observed in the lumbar sections of the spinal cord compared to the cervical sections. The mean density of GFAP positive astrocytes was found to be increased in the group K1 and K2 compared to group C in both cervical and spinal cord sections (Figure 5). Group K2 treated with high dose of KA showed a higher mean density of GFAP positive cells compared to group K1. A statistically significant increase (One way ANOVA, Post hoc Bonferroni, p<0.05) in mean density of GFAP positive astrocytes was observed in the lumbar sections of group K2 compared to Group C (Figure 5). Qualitative analysis of spinal cord sections under NFP
immunostaining revealed an increase in the NFP expression in the form of brown-coloured filaments around the motor neurons in the ventral gray horn in both Group K1 and Group K2 (Figure 4, NFP-K1, NFP-K2). The lumbar sections of the spinal cord exhibited a higher density of NFP compared to the cervical sections in both the experimental groups.

**Estimation of SOD**

Mean SOD activity in units per mg of the spinal cord homogenate in both cervical and lumbar regions was increased in group K1 and K2 compared to the SOD activity in control group C (Figure 6). One-way ANOVA analysis showed significant increase in mean SOD activity (Post Hoc Bonferroni, p<0.05) in spinal cord homogenate of both cervical and lumbar regions in group K1 compared to the SOD activity of control group C (Figure 6).

**Discussion**

Intra-cisternal administration of sub-toxic dose of KA in the male Wistar rats displayed signs of motor dysfunction and sensory impairment. The behavioral changes in the form of anxiety and a tendency to remain seated at the corner of the cage were observed in the rats receiving KA. Being a non-degradable analog of glutamate, the seizure activity was most common observation following intravenous, intraperitoneal, intranasal injections or microinjection into the brain of KA [5]. However, the seizure activity was not prominently observed in this study as the doses of KA used were low (50 and 150 fmol/day). The open-field observation for the locomotor activity for 14 days following intra-cisternal administration of KA showed an initial hyperactivity for first 48 hours which was followed by a reduction in mean action time which was not statistically significant. In a previous study, KA administration was found to impair the food pellet handling ability of the rats [21]. Contraction of a skeletal muscle depends on the neuromuscular transmission via activation of the spinal motor neuron. Previous in vivo and in vitro studies confirmed that motor neurons were highly susceptible to chronic AMPA/kainate receptor-mediated injury [22]. Thus, when the spinal motor neuron was injured by KA, neuromuscular transmission was unable to proceed. Glutamate-mediated neuronal excitation was described as one of the factors in the mechanism of neurodegenerative disorders such as ALS. In this study, intra-cisternal administration of very low sub-toxic dose of KA, failed to produce significant reduction of movement.

The threshold for sensation of pain towards an increase in the temperature (mean latency time
for licking of the paws in Hot Plate test) was increased immediately after KA administration. On the 14th day after the intra-cisternal administration of sub-toxic low dose of KA, the mean latency time showed no significant changes in the KA treated groups, compared to the control group. KA, a glutamate analogue, plays a significant role in the onset of post-injury pain-related behavior [23]. Depending on the route of KA administration, observation of both hyperalgesia and hypoalgesia has been shown in separate studies. Nociceptive primary afferent C-fibers express a subset of glutamate receptors that are sensitive to KA and are responsible for hyperalgesia produced by the intra-peritoneal or subcutaneous administration of KA. A previous study found that the intra-cisternal injection of KA into the cerebrospinal fluid, caused analgesia [24]. A European multicentre study found no difference between the subgroups of Amyotrophic Lateral Sclerosis patients with normal versus abnormal sensory nerve conduction findings with respect to age, duration and region of onset [25].

Nissl bodies are present in the cell body of neurons and they are composed of the rough endoplasmic reticulum. As observed in Nissl-stained spinal cord sections, the present study observed the features of neuronal damage in spinal motor neurons of the rats treated with both low and high dose of KA. KA produced an influx of Na\textsuperscript{+} ions and Cl\textsuperscript{-} ions which destabilised the intracellular Ca\textsuperscript{2+} ion homeostasis. The resulting detrimental biochemical events caused the neuronal damage. Morphological changes in the damaged neurons observed in the previous study [26] were neuronal shrinkage, chromatolysis, and dendritic swelling. In the damaged neurons, observed in the KA treated animals, the nuclei were pyknotic and the Nissl granules were lost with the shrinkage of the cell body.

Kainic acid is a potent glutamate analogue and it was used as a model of glutamate excitotoxicity study [27]. Glutamate excitotoxicity was identified as a type of CNS injury which caused the astrocytes to undergo hypertrophy, hyperplasia and induced reactive astrogliosis [5]. Reactive astrocytes are essential for wound healing and preservation of function of neurons. Using a mouse model, Faulkner et al. [28] demonstrated with thymidine kinase that dividing astrocytes may be involved in the survival of motor neurons during the spinal cord injury. It was found that the astrocyte cell division reaches the peak during 2 to 5 days after stab injuries of spinal cord [29]. GFAP is one of the essential factors released by astrocytes for the...
survival of neurons and wound healing. GFAP has a protective effect towards an insult to the CNS and increased GFAP expression was associated with reduced lesion volume and substantially increased white matter preservation [30]. From both cervical and lumbar sections of the spinal cord, the present study observed an increase in the GFAP positive astrocytes in group K1 and K2, compared to the control group. A significant increase in the mean count of GFAP positive astrocytes was found over the lumbar part of the spinal cord in the group of rats treated with 150 fmol/day of KA. The increase in GFAP production was considered a hallmark of astrogliosis in ALS [31] and an early biomarker of neurotoxicity [32].

NFP has been recognised as a well-known marker for neuronal damage as well as regeneration. Therefore large amount of NFP and its subunits were released as a result of axonal loss following CNS injury, such as excitotoxicity following KA administration. Pathologic accumulation of NFP both within spheroids of the proximal axon and within inclusions of motor neuron somata is a hallmark of neurodegeneration in ALS [33]. NFP, in the form of neurofibrillary tangles, was also seen in other neurodegenerative diseases like Alzheimer's Disease and Parkinsonism. Neurons expressing high somatodendritic levels of neurofilament were found to be vulnerable to neurodegenerative changes in the spinal cord in the mouse model of ALS [34]. An increase in the NFP expression in the axonal fibres around the motor neurons was observed in the present study in lumbar and cervical spinal cord sections of the rats exposed to KA.

The levels of SOD and Catalase, indicating oxidative stress, were increased significantly after KA administration [35]. KA was found to induce neurodegeneration and an increase in the free superoxide radical production resulting in an increase in SOD activity. Post-mortem brain tissue from ALS patients showed increased oxidative damage to the proteins [36]. Compared to the 150 fmol/day KA treatment, the effect of 50 fmol/day KA treatment on the SOD activity of the spinal cord homogenate was significantly higher compared to the control group. Djukic et al. 2012 [37] observed an increased oxidative stress only during early hours following Paraquat induced neurotoxicity and explained the protective role of glutathione and SOD in preventing early stage of neurotoxicity. The present study found relatively higher level of the SOD activity in the cervical part of the spinal cord in both the experimental groups exposed to intra-cisternal administration of KA compared to the lumbar part.

Conclusion

Intra-cisternal administration of kainic acid, a potent glutamate agonist was used to study the changes in the morphology of neurons and glial cells of the spinal cord as well as functional evaluation of the movement and nociception. Very low doses of KA (50 and 150 fmol/day) were used. The locomotor activity in the parameter of observed mean action time remained reduced on 14th day after administration of KA only in rats treated with 150 fmol/day. Although no KA-antagonist was used to observe the reversal of the motor and sensory impairment, the absence of any appreciable change in the motor and sensory tests in the control animals treated with normal saline proved the specific effects of KA. Increased GFAP expression, prominent neurofilament protein and increased SOD activity in lumbar and cervical parts of the spinal cord indicated persistent neuronal damage even after 2nd week of the administration of KA. It can be concluded that the spinal cord damage with some features similar to ALS can be produced by low dose intra-cisternal administration of KA.

Acknowledgements

The authors would like to thank the Research and Ethics Committee of International Medical University, Malaysia for the grant to carry out this research project.

Disclosure of conflict of interest

The authors report no conflict of interest.

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