

The Effect of long-term rapamycin administration on intracellular calcium quantification and RhoA activity in kainic acid epilepsy model

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Epilepsy affects around 50 million people worldwide, with 30% of them being refractory epilepsy. This shows that there is still a need for novel anti-seizure medication that have different mechanisms. One of the most common types of refractory epilepsy is temporal lobe epilepsy. Among the several effects of seizures on neurons is an increase in intracellular Ca^{2+} and activation of RhoA. Rapamycin is mTORC1 inhibitor, but long-term exposure to rapamycin (>18 hours) could also inhibit mTORC2. RhoA signaling pathway is regulated through the mTORC2 pathway; thus we hypothesized that long-term exposure to rapamycin could inhibit intracellular Ca^{2+} and RhoA activity as one of the mTORC2 downstream proteins, in a temporal lobe epilepsy model. This study used organotypic hippocampal slice cultures (OHSC) which were exposed to 20 nM rapamycin treatment for 3, 5, 8, and 10 days after induction of epilepsy by 7 μ M kainic acid administration for 48 hours. Intracellular calcium concentration was observed using CLSM and RhoA activity with western blot. The results obtained from this research were long-term administration of rapamycin can decrease intracellular calcium concentration and RhoA activity in OHSC models of epilepsy induced by kainic acid, with the most effective duration is 5 days of exposure to rapamycin.

Keywords: Rapamycin. Calcium. RhoA activity. Organotypic hippocampal slice culture. Epilepsy. Kainic acid.

INTRODUCTION

Epilepsy is a chronic disorder of the brain characterized by recurrent seizures due to paroxysmal neurologic events arising from excessive electrical discharges in the brain (Moalong *et al.*, 2021). In 2019, epilepsy is 0.5% of the global burden of disease, with 50 million people suffering from epilepsy worldwide. Besides that, WHO estimates that the number of disability-adjusted life years

(DALYs) caused by epilepsy is 13 million/year (WHO, 2019). As of 2018, it was stated that there were 30% of drug-refractory epilepsy patients, namely epileptic patients still experienced seizures, even increased seizure frequency and cognitive decline, despite taking Anti-Seizure Medication (ASM) (Magalhães *et al.*, 2018; Hodges, Lugo, 2020). One of the most common types of refractory epilepsy is temporal lobe epilepsy (TLE). In this type of epilepsy, hippocampal atrophy was found on MRI, which is then known to correlate with hippocampal sclerosis in the TLE study (Coan *et al.*, 2004).

It has recently been hypothesized that increasing intracellular Ca^{2+} levels above normal but below

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excitotoxicity levels will lead to changes in neural plasticity and will cause epilepsy if it occurs continuously (Zhang *et al.*, 2019). There has been a study showing that increased intracellular Ca^{2+} also causes activation of RhoA (Semenova *et al.*, 2007). RhoA belongs to the Ras superfamily of G proteins that are expressed in a variety of cells including neurons and astrocytes (Wang, Ren, Zheng, 2022). RhoA is also known to play a role in inhibiting neuronal regeneration, synapse remodeling, and altering the structural framework of neuronal networks during pathological conditions such as epilepsy (Yuan *et al.*, 2010). In a TLE model study, increased RhoA activity was also found and caused neuronal network remodeling (Yuan *et al.*, 2010). RhoA is activated in the hippocampus of the kainic acid-induced epilepsy model, and its inhibition with fasudil can protect neurites from injury due to the administration of kainic acid (Wang, Ren, Zheng, 2022; Xiang *et al.*, 2021). Increased RhoA expression was also found in brain samples from epilepsy patients (Yuan *et al.*, 2010).

The RhoA signaling pathway is regulated through the mTORC2 pathway (Chen *et al.*, 2018). mTOR is a serine/threonine kinase that has long been known to play an important role in the regulation of metabolism and cell growth, proliferation of adaptive immune function, and cell death process (Switon *et al.*, 2017; Weichhart, 2012). This protein consists of two multicomplexes, namely mTORC1 and mTORC2 (Switon *et al.*, 2017; Weichhart, 2012). Phosphoinositide 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) signaling activity has been shown to affect the brain tissue of individuals with epilepsy in several genetic studies or in vivo epilepsy models (Hodges, Lugo, 2020). Several things that are thought to cause epileptogenesis are changes in the mechanism of synapse plasticity, cell proliferation, and ion channel protein expression that occur due to activation of mTOR signaling in seizure conditions (Hodges, Lugo, 2020).

Rapamycin is a macrolide that can inhibit mTORC (Mannick, Lamming, 2023). However, it was later discovered that long-term exposure to rapamycin (>18 hours) could inhibit mTORC2 (Lipton, Sahin, 2014). Thus,

rapamycin is both a mTORC1 inhibitor and a mTORC2 inhibitor upon long-term exposure. We hypothesized in this study that long-term exposure of rapamycin could inhibit intracellular calcium and RhoA activity as one of the mTORC2 downstream proteins, in a temporal lobe epilepsy model.

MATERIAL AND METHODS

Organotypic Hippocampal Slices Culture

Hippocampal slice culture was performed according to the method described by Blazejczk *et al.*, 2017 and has received ethical approval from the Health Research Ethics Commission, Faculty of Medicine, Universitas Brawijaya. First, decapitation was carried out on *Rattus norvegicus* Wistar strain aged 7-10 days without using anesthesia with previous disinfection using 70% alcohol and cervical dislocation. The brain was isolated and incubated for 10 minutes in a slicing medium consisting of 24 g HEPES (Sigma, H3375, Lot 011M5434) dissolved in 10 mL EBSS (Sigma, Lot RNBG8086) for 10 minutes on ice with continuous aeration of 95% O_2 and 5% CO_2 . After that, The hippocampus was isolated in a slicing medium under a stereo microscope (Olympus) to include the dentate gyrus, CA1, and CA3 region of the hippocampus. The hippocampus was sliced with a manual tissue slicer (Stoetting Tissue Slicer 51425) for coronal sectioning at 350 μm . The slices were gently separated and placed into 6-wells cell culture plate (Biologix) which had been given millicell cell culture inserts (0.4 m, diameter 30 mm, Merck, Lot: R9JA56969), and above it was placed a millipore membrane (LCR Filter type 0.45 m PTFE Membrane, 13 mm, Merck, Lot: R9JA55940) which had absorbed culture medium. A culture medium containing 0.25 mL Penicillin-Streptomycin (Gibco, Ref15140-122, Lot 1665606, 100 mL), 5 mL Horse Serum (Abcam ab7484, Lot GR3217249-1, 25 mL), 0, 25 mL Amphotericin B (Gibco, Lot 2090190), 12.5 mL Minimum Essential Medium Eagle (MEM) (Sigma, M0769-10x1L, Lot SLBS6945), 4.5 mL Earle's Balanced Salt Solution (EBSS) (Sigma, Lot RNBG8086, 500 mL), 1.25 mL EBSS + 6.5 grams D-glucose (Dextrose) (Abcam,

ab143108, Lot GR186597-6, 10 mg) in 50 mL EBSS. The OHSCs were then incubated at 37°C with a 5% CO₂ incubator and the culture medium was replaced every 2 days. Administration of 7 µM kainic acid (Abcam, ab120100) in groups K⁺, D3, D5, D8, and D10 was performed on 7-11 DIV by bath application dissolved in culture medium for 48 hours. The next step was to administer 20 nM rapamycin (MedChemExpress, HY-10219) by bath application to D3, D5, D8, and D10 groups for 3, 5, 8, and 10 days, while the K⁺ group was given DMSO as a solvent for rapamycin. All of the above activities are carried out with sterile procedures in a laminar air flow (Faster).

Calcium Labelling

Intracellular Ca²⁺ was evaluated with the immunofluorescence method based on the standard procedure in Fluo-4 Assay Kit (Calcium) (Abcam: ab228555; Lot GR3249725-1). Fluo-4 dye solution was prepared by mixing 20 µL of Fluo-4 AM stock solution with 1x Assay Buffer consisting of 1 mL of 10X F127 Plus and 9 mL of HHBS (Hank's Balance Salt Solution) buffer in a dark room. The OHSCs were washed with ice-cold PBS three times and the prepared solution was added still in the dark room. Incubation with this solution for 1 hour in a 37° C incubator with 5% CO₂. After the incubation process was completed, the samples were washed with HHBS three times and followed by observations under a confocal laser scanning microscope (CLSM) (Olympus type FV1000) at Ex/Em = 490/525 nm intensity. Quantification of intracellular Ca²⁺ expression was performed using Olympus Fluoview Ver 4.2a software based on fluorescence intensity in the hippocampus.

Protein Isolation

Proteins were isolated using 18 slices of OHSC culture for one sample group. First, a clean glass dish was placed on ice, and then 18 slices of OHSC tissue were

placed and washed with ice-cold PBS three times. After that, 200 µL of lysis buffer (Cytoskeleton, Part #CLB01) was given, which had previously been mixed with a 2 µl protease inhibitor cocktail (Cytoskeleton, Cat. #PIC02). The tissues were homogenized using a cell scraper while remaining on ice. Once completely smooth, transfer the tissue lysate into a new tube and centrifugate at 10,000 x g, 4 °C for 1 minute immediately. Transfer the supernatant into another tube and the supernatant was transferred to a new tube. Protein concentration measurement was performed using a nanodrop spectrophotometer.

Immunoprecipitation

Immunoprecipitation was conducted based on the standard protocol from the Rho Activation Assay Biochem Kit from Cytoskeleton (Part # CLB01). First, 0.5 mL of tissue lysate was loaded with GTP which was added to 35 µL of loading buffer. Then immediately added 5 µl of GTPγS and incubated at room temperature for 15 minutes with gentle rotation. Then stop the reaction by moving the tube to 4° C and adding 50 µl of stop buffer.

Pull Down Assay

Pull-down assay was conducted based on the Rho Activation Assay Biochem Kit from Cytoskeleton (Part # CLB01). Protein concentration was equalized for each sample group and Rhotekin-RBD beads was added to each sample. After that, the samples were incubated at 4° C above the rotator for 1 hour. Then, to precipitate rhotekin-RBD beads, centrifugation was carried out at 3 – 5000 x g at 4° C for 1 minute and the supernatant was separated. The beads were washed once with 500 µl of wash buffer each. To precipitate the formation of rhotekin-RBD beads again, centrifugation was carried out at 3-5000 x g at 4° C for 3 minutes and the supernatant was separated again to another tube and 2x Laemmli sample buffer 10 µL was added to each tube. After that, the beads were resuspended and boiled for 2 minutes.

SDS-PAGE and Western Blot

SDS-PAGE was performed by heating the protein sample at 100° C for 5 minutes in a buffer solution containing 5 mM Tris pH 6.8; 5% 2-mercapto ethanol; 2.5% x/v sodium dodecyl sulfate, 10% v/v glycerol using bromophenol blue as a tracer color. 12.5 mini slab gel with 15% separating gel and 4% staking gel were selected. After the SDS-PAGE procedure was done, the samples were transferred to a nitrocellulose membrane. Non-specific binding was blocked by incubation in 5% TBS-Skim Milk overnight at 4° C. For immunodetection, the membrane was incubated in RhoA primary antibodies (1:500, Cytoskeleton, Part #CLB01) in TBS-Skim milk for 2 hours at room temperature and followed by washing with TBS-Tween 20.05% for 3 x 5 minutes. After that, the samples were in a solution of secondary antibody (IgG anti-mouse-HRP 1:2000, Ab6725) for 2 hours at room temperature.

Band Visualization

Chemiluminescence method was used for band visualization. A detection reagent consisting of 150 mL peroxide solution (Thermo Scientific, Lot # VB295971, 250 mL) and 150 mL luminol enhancer solution (Thermo Scientific, Lot #VB299180, 250 mL) was added to the nitrocellulose membrane in a dark room with gentle rotation for 30 minutes. The reaction was stopped by distilled water and the results were immediately recorded with ImageQuant LAS 500 (GE Healthcare). The thickness of the protein band was quantified using the ImageJ software program.

Statistical Analysis

Data were expressed as means $\pm$ SD. For multiple variable comparisons, data were analyzed by One-Way Analysis of Variance (ANOVA) followed by an Least Significant Difference (LSD) test. For the correlation test, data was analyzed using Pearson's test. Statistical analysis was performed using SPSS for Windows 25 software (IBM), $p < 0.05$ was considered statistically significant.

RESULTS

Long-Term Rapamycin Treatment Lower Intracellular Ca^{2+} Concentration in OHSC Model of Epilepsy

Long-term rapamycin treatment can significantly lower intracellular Ca^{2+} concentration. Figure 1A shows a comparison of intracellular Ca^{2+} immunofluorescence which qualitatively represents Ca^{2+} levels. The longer the rapamycin treatment, the lower the fluorescence results. The D10 group showed the lowest fluorescence, followed by the D8 group, negative control, D5 group, and D3 group. The highest fluorescence was in the epilepsy group. The expression of intracellular Ca^{2+} fluorescence was then quantified using ImageJ software by calculating the intensity of the resulting color which interprets the intracellular Ca^{2+} concentration. Figure 1B shows the mean and standard deviation of intracellular Ca^{2+} concentrations from four repetitions of each group. The D10 group had the lowest average intracellular Ca^{2+} concentration, with a percentage reduction compared to the epilepsy group reaching 55.91%. This study also proves that exposure to 7 μM kainic acid for 48 hours in OHSC can increase calcium concentration significantly compared to negative control.

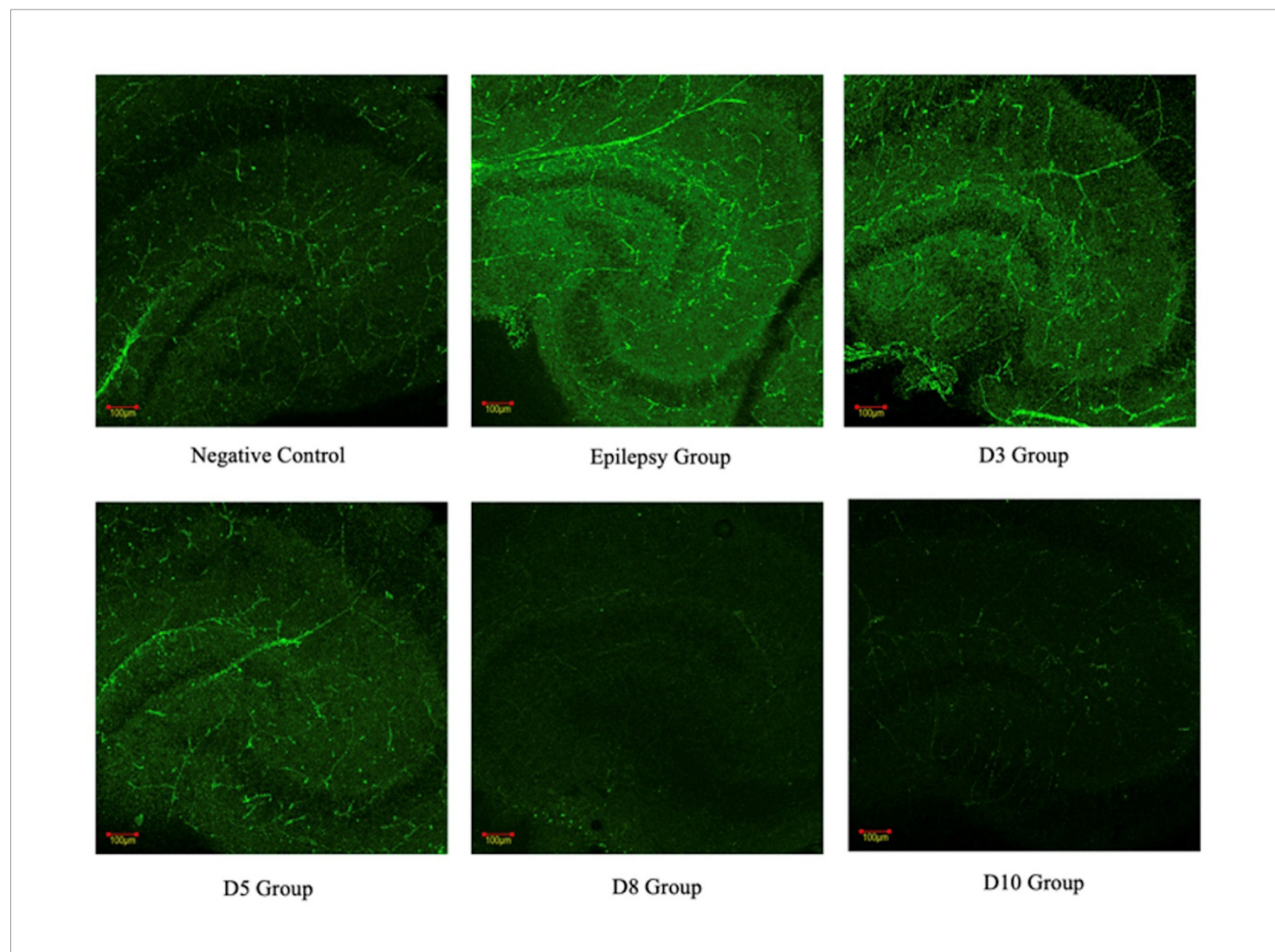


FIGURE 1 A-B - Evaluation and Analysis of Intracellular Ca^{2+} Concentration Between Groups. Intracellular Ca^{2+} expression of OHSC between groups under confocal laser scanning microscope (CLSM) is represented as a green fluorescence color. (A) The longer the duration of rapamycin treatment, the lower the intracellular Ca^{2+} expression. (B) The longer the duration of rapamycin treatment, also showed a decreased mean intracellular Ca^{2+} concentration. There was a significant decrease in the negative control and all treatment groups against the epilepsy group. *Compared to the epilepsy group, $p < 0.05$.

Long-Term Rapamycin Treatment Lower RhoA Activity in OHSC Model of Epilepsy

Long-term rapamycin treatment also significantly lower intracellular Ca^{2+} concentration. In Figure 2A, the bands appear with a molecular weight of + 21 kDa which corresponds to the molecular weight of the RhoA protein. The D10 group showed the lowest band thickness, followed by the D8 group, negative control, D3 group, and

D5 group. The highest band thickness was in the epilepsy group. The thickness of the band was then quantified using ImageJ software by calculating the color density of the resulting band and interprets the intracellular Ca^{2+} concentration RhoA activity. Figure 2B shows the mean and standard deviation of RhoA activity from four repetitions of each group. The results of the following RhoA activity had similar results to intracellular Ca^{2+} concentrations, namely D8 and D10 groups had lower

band thickness than the negative control group. There was a difference in the D5 group which had higher RhoA activity than D3 group. The 10D group of rapamycin treatment has the lowest RhoA activity average, with a reduction percentage compared to the epilepsy group

reaching 69.14%. This study also proves that exposure to 7 μ M kainic acid for 48 hours in OHSC can increase RhoA activity significantly compared to negative control. Intracellular Ca^{2+} decrement was correlated with RhoA activity ($R^2 = 0.6323$).

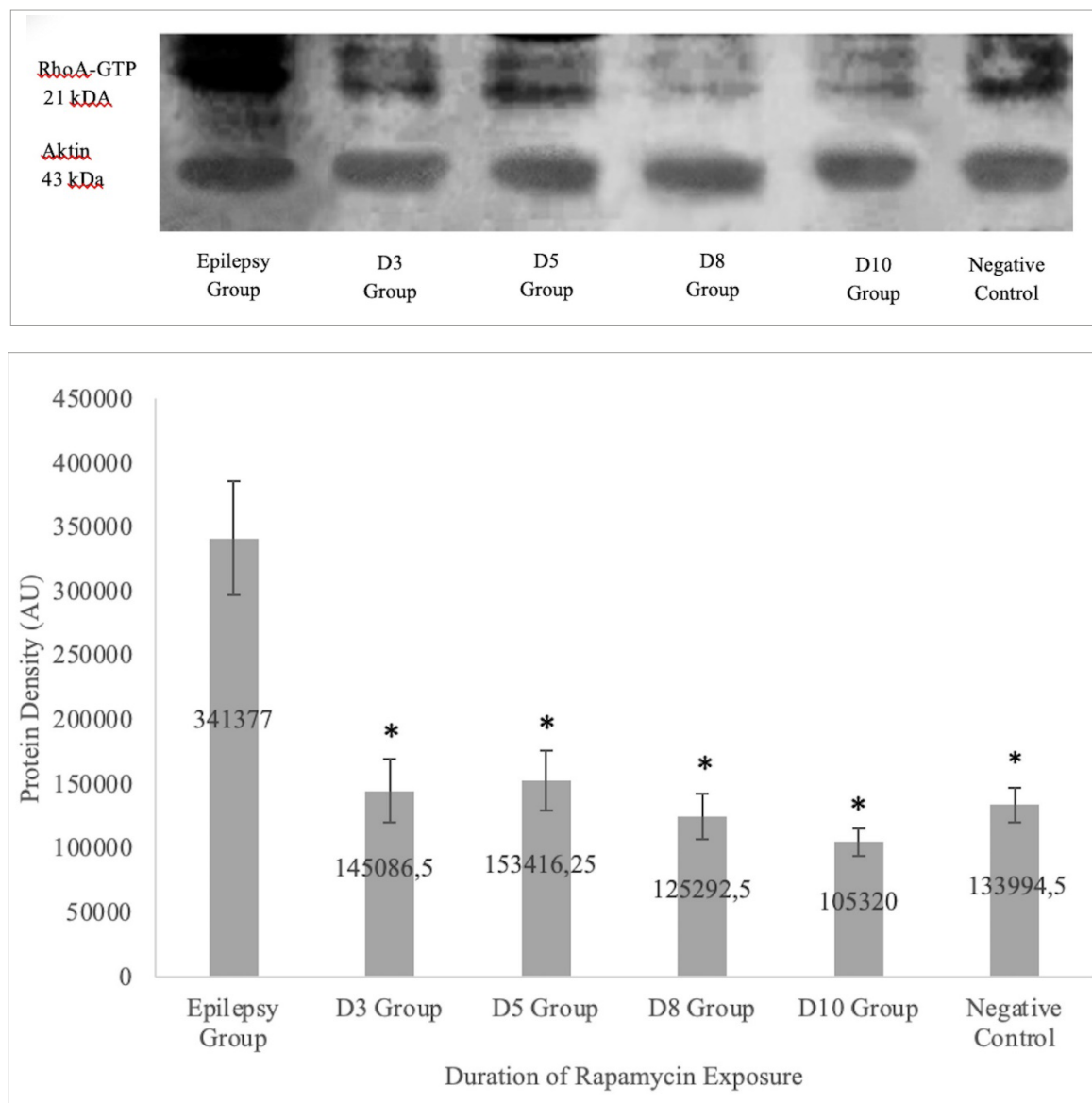


FIGURE 2 A-B - Evaluation and Analysis of RhoA Activity Between Groups. Western blot results of RhoA activity. (A) The longer the duration of rapamycin treatment, the lower the RhoA activity in treatment group, except D5 group. (B) The longer the duration of rapamycin treatment also showed a decreased mean of RhoA activity, except D5 group. There was a significant decrease in the negative control and all treatment groups against the epilepsy group also showed a decreased mean of RhoA activity. *Compared to the epilepsy group, $p < 0.05$.

DISCUSSION

In this study, the OHSC model of epilepsy was carried out by induction of kainic acid for 48 hours. Kainic acid as a seizure-inducing agent has been used since the 1980s to identify excess excitatory action in neurons (Ben-Ari, 2010). The first result in this study indicates kainic acid can increase calcium concentration and RhoA activity significantly. Activation of KA receptors by kainic acid will produce excess glutamate neurotransmitters which will cause sustained epileptic activity in the hippocampus or cause neuronal death (Ye *et al.*, 2019). Various TLE studies with OHSC have used kainic acid as a seizure induction agent. Research by Routbort *et al.* (1999) and Bausch, McNamara (2004) used 6-7 μM kainic acid which was incubated in cultures for 48 hours to observe the mossy fiber sprouting which can cause neuronal hyperexcitability. KA treatment has been proven in genetic and experimental studies that affect neuronal plasticity and morphological changes in dendrites, partly through the activation of the RhoA/Rho kinase pathway which increases cofilin (Xiang *et al.*, 2021; Sharma *et al.*, 2009). Besides that, the induction of kainic acid has a direct role in causing Ca^{2+} influx, so it will increase the intracellular Ca^{2+} concentration (Zhang, Zhu, 2011; Banerjee, Ghosh, Mondal, 2012). However, one of the limitations of OHSC is the minimal extracellular space, so concentrations in the intracellular area are often observed (Zhang, Zhu, 2011). One approach that can be done to overcome that limitation is to use fluorescent dyes which are membrane permeable. Where an increase in staining will indicate an increase in the observed ion concentration (Banerjee, Ghosh, Mondal, 2012).

The second result is long-term rapamycin treatment significantly reduces intracellular Ca^{2+} , while the D5 group has an intracellular Ca^{2+} concentration close to the negative control group. Rapamycin has been known to bind to endogenous FKBP-12 which binds to intracellular Ca^{2+} receptor channel, thus inhibition of FKBP12 by rapamycin will lead to direct inhibition of intracellular Ca^{2+} (Pitkänen Galanopoulou, Moshé, 2017; Terashima *et al.*, 2000). In a previous study by Ruan *et al.* (2008) and

Kaftan, Marks, Ehrlich (1996) showed that rapamycin modification in the mTOR binding region can inhibit the L-type Ca^{2+} channel. So, inhibition of Ca^{2+} channels will be able to inhibit the over-activation of glutamate receptors that experience over-activation in epileptic conditions.

The third result is long-term rapamycin treatment significantly reduces RhoA activity, while the D5 group has a RhoA activity close to the negative control group. Inhibition of RhoA has the potential to inhibit dendritic structure formation after seizures which has the clinical potential to prevent or reverse seizure-induced cognitive impairment (Xiang *et al.*, 2021). In a previous study, it was found that long-term rapamycin exposure in an animal model of kainic acid-induced epilepsy showed its effectiveness in inhibiting kainic acid induction and in causing cell death, neurogenesis, axonal sprouting, and spontaneous seizures (Switon *et al.*, 2017). The mechanism that occurs in long-term rapamycin exposure is the interaction of FKBP12-rapamycin with Rictor, the largest free fraction of mTORC2 molecules (Weichhart, 2012). Rictor which binds to FKBP12-rapamycin will be inhibited from joining mTOR in forming mTORC2 which will cause inhibition of the Akt/PKB signaling process, one of the most influential signaling pathways for epilepsy occurrence (Weichhart, 2012). Recently, there have been no studies observing the effect of long-term rapamycin administration on RhoA activity in OHSC models of epilepsy, both in vivo and in vitro studies. A study conducted by Liu *et al.* (2010) in cell cultures of Human Rhabdomyosarcoma Rh30 and Ewing Sarcoma Rh1 exposed to rapamycin 100 ng/ml for 24 hours showed a decrease in RhoA protein expression which is thought to occur through inhibition of protein synthesis. Meanwhile, the increase in RhoA activity in the D5 group compared to the D3 group in this study was probably due to exposure to rapamycin requiring a certain amount of time to stabilize in reducing RhoA activity. In this study, the minimum time expected to reduce RhoA activity was in the D8 group. This is what causes the D8 group to show a decrease in RhoA activity under negative control. The previous study conducted by Sarbassov *et al.* (2006) inhibited the

formation of Rictor through inhibition of the Akt/PKB signaling pathway by rapamycin upon exposure after 72 hours to the HeLa and PC3 cell lines.

The last result is a positive correlation between intracellular calcium concentration and RhoA activity. The increase in the percentage of intracellular Ca^{2+} concentration was also accompanied by an increase in the percentage of RhoA protein density. In previous studies, it was stated that an increase in intracellular Ca^{2+} led to an increase in RhoA activity (Yuan *et al.*, 2010).

Currently, two classes of ASMs are commonly used, namely conventional and new generation (Katzung, Trevor, 2015). The mechanism of conventional ASM groups is by prolonged inactivation of ion channels Na^+ , Ca^{2+} , K^+ , or those that generally work to inhibit the excitatory neurotransmitter glutamate which will increase steadily in epilepsy (Katzung, Trevor, 2015; Engelborghs, D'hooge, De Deyn, 2000). The mechanism of the new class of ASM has three basic mechanisms, in the form of increased GABAergic transmission (inhibition), decreased excitation transmission (usually glutamatergic), or modification of ionic conductance (Katzung, Trevor, 2015). This study has the hypothesis that the inhibition of ion channels that affect epilepsy events and the inhibition of proteins that cause epileptogenesis will be able to inhibit seizure frequency in cases of refractory epilepsy. Ca^{2+} influx during epilepsy is successfully inhibited by long-term rapamycin exposure as evidenced by a decrease in intracellular Ca^{2+} concentration. On the other hand, RhoA, which is a protein that causes epileptogenesis, has been shown to decrease with long-term rapamycin exposure. Thus, long-term rapamycin administration has the potential to become a novel anti-epileptic agent for refractory epilepsy that can directly inhibit Ca^{2+} influx and RhoA activity, which will inhibit the hyperexcitability of neurons that cause epileptogenesis.

From this study, it was concluded that long-term rapamycin administration could significantly reduce intracellular Ca^{2+} concentration and RhoA activity in the Organotypic Hippocampal Slice Culture model of epilepsy induced by kainic acid in OHSC, this showed potential for long-term rapamycin as a novel therapy for

epilepsy. In addition, this study also found that the most effective duration of rapamycin 20 nM exposure in the OHSC model of epilepsy induced by kainic acid was 5 days, this can be considered as a maximum dose of rapamycin administration in this model. Further studies are needed to evaluate the long-term toxicity effects of rapamycin as a candidate for a novel epilepsy drug. In addition, it is necessary to conduct similar studies using other models of epilepsy and studies that compare the effectiveness of long-term rapamycin administration compared to existing epilepsy drugs.

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