ORIGINAL ARTICLE

Relationship Between *Smn1* and *Casp3* Gene Expression in Kainate-Induced Spinal Cord Injury Model

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ABSTRACT

Background: Trauma to the spinal cord, known as spinal cord injury (SCI), can result in paralysis and other motor impairments. SCI causes significant cellular damage via glutamate excitotoxicity that ultimately results in cell death. The survival motor neuron (SMN) is one of the regulators of apoptosis and mutation of the gene causes spinal muscular dystrophy (SMA). Although cells in SCI undergo apoptosis, the interplay between *Smn1*, gene coding for SMN, and caspase 3 (*Casp3*) remained unknown. Therefore, this study aimed to determine the relationship between *Smn1* and *Casp3* in kainite induced in-vitro SCI model. **Methodology:** NSC-34 motor neuron-like cell line, was differentiated into neurons using 1 µM of retinoic acid prior to excitotoxicity induction by kainic acid for 48 hours. Expression of *Smn1* and *Casp3* were measured using quantitative polymerase chain reaction (qPCR). Data was analysed by one-way ANOVA using GraphPad Prism. **Results**: Approximately 40% of cells die when 0.5 and 1 mM of kainic acid was added. The expression of *Smn1* was significantly elevated (3-fold) in 0.5 and 1 mM kainic acid. However, there is no significant change for the *Casp3* expression, suggesting that apoptosis was not activated in kainic acid-induced SCI. **Conclusion:** This research contributes to our understanding of the molecular landscape of spinal cord injuries, indicating that Casp-3 independent mechanisms may exist in *Smn1*-expressed SCI induced by kainate excitotoxicity.
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Keywords: Apoptosis, cell death, chemical injury, kainite excitotoxicity, *smn1*, spinal cord injury

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INTRODUCTION

Spinal cord injuries (SCI) constitute a significant health challenge in Malaysia, predominantly stemming from vehicular accidents and falls, with a disproportionate impact on young males aged 16 to 30 (1), a demographic that often experiences life-altering consequences such as paraplegia. Despite the gravity of SCI and its profound implications, including paralysis, there is a lamentable absence of a definitive cure for this condition (2). The injury process unfolds in two distinct phases: the primary injury occurring immediately after trauma and the subsequent secondary injury marked by cellular changes that contribute to the progression of damage beyond the initial impact. During the second phases of SCI, there is an imbalance in glutamate levels, leading to excessive excitation and persistent excitotoxicity of glutamate receptors, which ultimately leads to the death of cells (3). Cell death via apoptosis, is one of the hallmarks of primary and secondary injuries (4). Apoptosis is a programmed cell death that is under strict regulation by the caspases, including caspase-3. Both internal and external stimuli can induce apoptosis, which causes cells to commit suicides.

An RNA-binding protein called survival motor neuron (SMN) plays a pivotal role in regulating apoptosis in spinal muscular atrophy (SMA) cell-based model (5). SMA is a genetic disorder characterized by the degeneration of motor neurons in the spinal cord, leading to muscle weakness and atrophy. *Smn1* gene, encoding for SMN, is ubiquitously expressed in the body and plays a vital role in DNA repair, pre-mRNA splicing, translation, mRNA trafficking, and in stress response (6). Furthermore, SMN has been implicated to have anti-apoptotic role in SMA (7). While SMN is predominantly recognized for its involvement in SMA,

emerging evidence suggests potential implications for its role in the pathophysiology of SCI. However, the relationship between *Smn1* and *Casp3* gene expressions in SCI remain poorly understood, presenting a critical gap in our knowledge. Therefore, this study aimed to unravel the relationship between *Smn1* and *Casp3* gene expression in an in-vitro SCI model.

MATERIALS AND METHODS

Cell Culture

The NSC-34 cell line was purchased from Cedarlane Laboratories The cell was revived and cultured in a T75 flask containing Dulbecco's modified Eagle's medium-F12 (DMEM-F12; Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, USA) and 1% of penicillin-streptomycin (Pen-strep; Thermo Fisher Scientific, USA). The cultures were incubated at 37°C in 5% of CO2. The media were replenished every 2 to 3 days, and sub-cultured once reached 70-80% confluency. The cultures were treated with trypsin-EDTA (Sigma-Aldrich, USA) to detach the cells from the flask.

Cell Differentiation

Poly-L-lysine (Sigma-Aldrich, USA) treated plates were used for the experiments. Approximately 20 000 and 200,000 of NSC-34 cells were plated in a 12-well and 6 well-plates, respectively. The cells were incubated at 37°C in 5% of CO_2 overnight before differentiation media, which consisted of DMEM-F12, 1% FBS, 1% pen-strep and 1% non-essential amino acid (NEAA; Sigma-Aldrich, USA). A differentiation agent, retinoic acid (RA; Merck, Germany) was added separately in each well at 1 µM concentration.

Chemical Injury via Kainic Acid

Differentiated NSC-34 cells were exposed to kainate receptor agonist, kainic acid (KA: Santa Cruz, USA), dissolved in differentiation medium for 48 hours. Two concentrations of KA (0.5 mM and 1 mM) were used, with a KA-free condition serving as the control. The analysis of the injury was determined by the cell death assay.

Cell Death Assay

The cultured cells were washed with culture medium after exposure to KA. To detect dead cells, two types of staining dyes were used: i) propidium iodide (PI), and ii) Hoechst staining (Thermo Fisher Scientific, USA), which stained the nucleus of all cells. Both PI and Hoechst were diluted in culture medium at a ratio of 1:200. The staining solution was added to each well. After 30 minutes of incubation, 4% paraformaldehyde was added to the cells for 10 minutes. The cells were washed in PBS before being images were captured using fluorescence microscopy (three x40 magnification images were captured per sample). The number of PI- and Hoechst-positive cells was determined using ImageJ.

Smn1 Primer Design Using NCBI Primer Blast

The initial step in this protocol involved utilizing the NCBI Primer-BLAST Pick Primers online tool, as previously reported (8). Primer sequences used are as follows: *Smn1* (Fw): GCTCCGTGGACCTCATTTC and Rev: GGGCCGTTGAATTTTAGACC; Actb (Fw) CATTGCTGACAGGATGCAGAAGG and Rev: TGCTGGAAGGTGGACAGTGAGG.

RNA Extraction, cDNA Synthesis and Quantitative PCR

RNA was extracted from samples using Monarch Total RNA Miniprep Kit (T2010S, New England Biolabs) according to the manufacturer's protocol. Total RNA (1 ug) was reverse transcribed into cDNA using LunaScript RT SuperMix Kit (E3010, New England Biolabs). Quantitative PCR (qPCR) was performed using Luna Universal qPCR Master Mix kit (M3003, New England Biolabs) using primers for *Smn1*, *Casp3* (Mm. PT.58.30114421 PrimeTimeTM predesigned qPCR assays, and Actb genes. The qPCR was performed using StepOne Plus Real Time PCR (Thermo Fisher Scientific) machine.

Statistical Analysis

The results were obtained from the observation through phase-contrast microscopy. For cell death assay, the percentage of healthy cells from the cell death assay were manually counted and confirmed using ImageJ (version 1.54p). The graph bar for the percentage of healthy cells versus the concentration of KA was produced by using Graphpad Prism (version 8). The quantitative data were expressed as mean ± standard error of the mean (SEM) or standard deviation (SD). Statistical significance was assessed using a one-way analysis of variance (ANOVA). A p-value less than or equal to 0.05 ($p \leq 0.05$) was considered as statistically significant.

RESULTS

Cell Differentiation Using Retinoic Acid

The NSC-34 cell line is a combination of neuroblastoma and motor neurons from the spinal cords of mouse embryos. To establish an in vitro SCI model, NSC-34 cells were differentiated in DMEM-F12 medium with the addition of differentiating agent, retinoic acid. NSC-34 cells cultured without retinoic acid serves as a negative control. Neurites development in differentiated NSC-34 was observed for 7 days. The results showed that upon treatment with 1 µM of retinoic acid, the number of sprouting neurites increased, and the neurites became more elongated as the period of differentiation increases from day 1 to day 7 (Fig. 1). On the day 1 of retinoic acid exposure, some of the NSC-34 cells started to develop protruding structures, whereas the cells in control condition retained rounded-shaped structure. After retinoic acid treatment, more cells with elongated protruding structures were observed on day 3 and became robust by day 7.

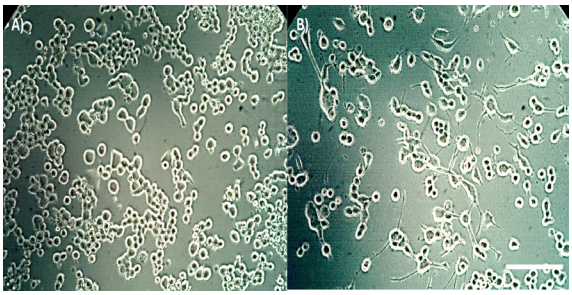

Fig. 1: NSC-34 cell differentiation. A) NSC-34 cells were cultured in serum-deprived media without retinoic acid. B) NSC-34 cells were cultured in differentiation media containing 1 µM of retinoic acid. The development of neurites was observed at day 7 under phase-contrast microscopy at 20x magnification.

Cell Injury Using Kainic Acid

On the seventh day of differentiation, differentiated NSC-34 cells were induced with KA at the following concentrations: 0, 0.5 and 1 mM. The cells were incubated with KA for 48 hours before being observed under phase-contrast microscopy (Fig. 2).

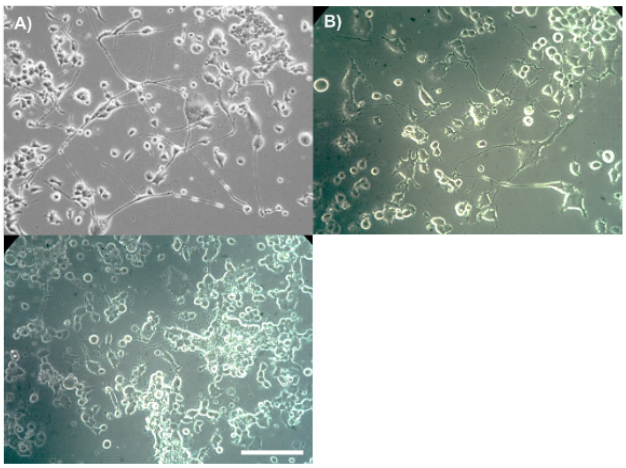

Fig. 2: Phase-contrast images of kainic acid-induced cells. A) Differentiated NSC-34 cells without kainic acid (control). Differentiated NSC-34 cells were induced with (B) 0.5 mM or (C)1 mM of kainic acid. The cells were observed under phase-contrast microscopy at 20x magnifications. Scale bar represents 20 µm.

Cell Death Assay

Propidium iodide (PI) was used to stain the dead cells after the injury. To study cell viability, the number of viable cells were calculated against the total number of all cells. The number of dead cells stained by PI versus the total number of cells were calculated as percentage (Fig. 3). Results show approximately 39% ±1.8984 and 39% ±6.8760 of cells died following treatment with 0.5 and 1 mM of kainic acid, respectively, indicating that at these concentrations were sufficient to promote injury thereby activating cell death mechanisms.

Expression of Survival Motor Neuron 1 And Caspase 3 In Injured Cells

NSC-34 cells were differentiated into neurons by retinoic acid before different concentrations of kainic acid (0, 0.5 and 1mM) were added to the cells for 48 hours in an incubator at 5% of CO_2 and 37°C. The expression of *Smn1* and *Casp3* (apoptosis marker) were measured against the house-keeping gene, beta actin (Actb). The qPCR result shows a significant elevation of *Smn1* (by three-fold) following treatment with 0.5 and 1 mM of kainic acid (Fig. 4a). In contrast, the expression of *Casp3* remained unchanged following treatment (p=0.4420) (Fig. 4b).

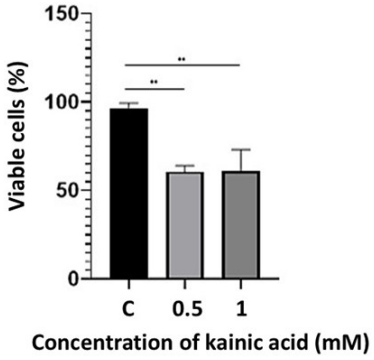

Fig. 3: Percentage of viable cells at different kainic acid concentrations. The percentage of healthy cells were counted by using ImageJ (n=3). Values are mean±SEM. The statistical analysis was performed using One-Way ANOVA followed by Dunnett's post-hoc test. *p≤0.05, **p≤0.01

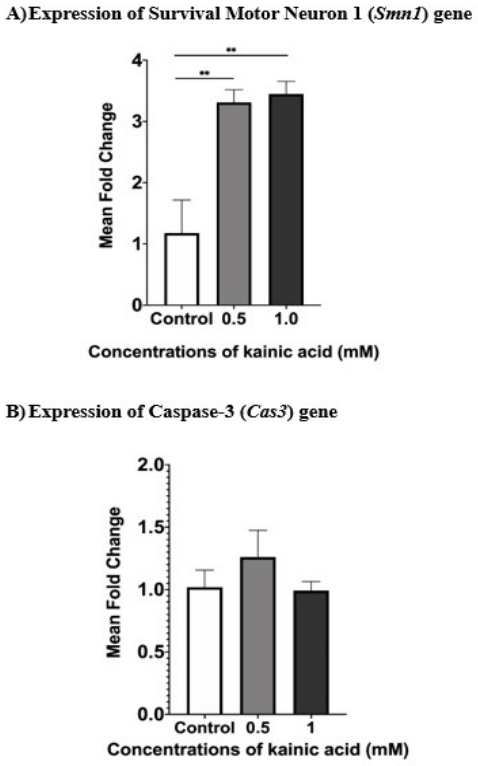

Fig. 4: Expression of Survival motor neuron 1 (*Smn1*) and Caspase 3 (*Casp3*) using quantitative RT-PCR. Values are mean + SEM (n=3). The statistical analysis was performed using one-way ANOVA followed by Dunnett's post-hoc test. **p≤0.01

DISCUSSION

SCI affects more than 900,000 people globally, each year (9). The primary causes of SCI are accidents that involve a significant impact or force on the spinal cord, such as motor vehicle accidents, falls from heights, sports- related incidents, and acts of violence (10).

During a traumatic event, the spinal cord can sustain direct trauma, resulting in various types of damage, including contusion (bruising), compression, laceration (tearing), or complete severance. The pathophysiology of SCI involves both primary and secondary phases of injury, each contributing to the overall neurological deficits observed in affected individuals. Primary injury occurs immediately following the initial impact to the spinal cord, while the secondary stage involves a more complex multitude of cellular and molecular events (11). This secondary injury involves glutamate excitotoxicity, calcium overload, mitochondrial dysfunction, and extensive cell death (programmed and nonprogrammed cell death) (12).

Treatment with Kainic-Acid Activates Cell Death

The differentiated NSC-34 cell lines were treated with various concentrations of kainic acid (0.5 mM and 1 mM) to induce cellular injury. The morphology of NSC-34 cells was observed 48 hours after the exposure to the KA-induced injury. Differentiated NSC-34 cells in control condition retained typical motor neuron-like structure with intact neurites, indicating healthy cells unaffected by kainic acid-induced injury. Clumps of dead cells formed in the presence of 0.5 and 1 mM kainic acid. The population of the cells started to deplete as the exposure prolonged to 48 hours. As more clumping of dead cells formed, the neurites also disintegrated and diminished, indicating cell death. Results of this study suggested that excessive activation of kainate receptor is one of the factors leading to neuronal death. Although several studies have been done to investigate the glutamate excitotoxicity on motor neuron-like cells, most studies were focusing on other ionotropic glutamate receptors, i.e. NMDA and AMPA, rather than kainate receptors (13, 14). Most of the research on kainate receptor excitotoxicity were done on the brain region and indicated that kainate receptor excitotoxicity plays a role in the pathogenicity of neurodegenerative disorders (15). Hence, the result of this study indicates that kainate receptor plays a key role in the mechanism of cell death in SCI.

Expression of Survival Motor Neuron 1 In Injured Cells

The survival motor neuron (SMN) protein, coded by *Smn1* gene, plays a critical role in cellular processes. SMN is ubiquitously expressed in the body and has a variety of roles ranging from splicing, autophagy, signal transduction, cellular stress response, and DNA repair (16). SMN has also been implicated in modulating neuron-specific apoptosis (17). *Casp3* expression was elevated in *Smn1*-depleted neurons in SMA in-vitro model, indicating SMN has a role in protecting cells from apoptotic cell death [5]. However, the precise mechanisms and significance of SMN in the context of SCI remain poorly understood, presenting a critical gap in our knowledge.

The qPCR analysis revealed that *Smn1* increased its expression following injury by 0.5 and 1 mM of kainic acid. This coincided with previous findings that showed that *Smn1* rose three-fold in subacute SCI animal model (18). This notable elevation of *Smn1* expression in-silico and in-vivo raises intriguing connections with the apoptosis process occurring in SCI. Apoptosis, or programmed cell death, is a complex cellular mechanism that plays a critical role in tissue homeostasis and injury response. The potential enhancement in *Smn1* gene activity suggests its involvement in mitigating or suppressing apoptosis in the context of SCI. However, our finding revealed no significant change of *Casp3* expression in injured cells despite elevated *Smn1* expression. Therefore, our preliminary findings indicate that the activation of *Smn1* gene occurs during injury, potentially serving as a safeguard against cell death that is unrelated to apoptosis, or through other unique roles.

Overall, these findings point towards a complex interplay between *Smn1* and *Casp3* genes in SCI. Although the research gave rise to positive findings, it also identified limitations that should be addressed in future investigations. Further studies should include western blotting to validate the changes to protein expression levels. Additional investigation into the precise molecular pathways of cell death triggered by different KA concentrations will yield important information on KA excitotoxicity in cell-based models.

CONCLUSION

In conclusion, the observed elevation in the level of *Smn1* gene following neuronal injury by kainic acid suggests a pivotal role for SMN in the physiological changes associated with SCI. This finding underscores the dynamic response of *Smn1* gene expression to the pathological conditions initiated by kainic acid, known for its role in excitotoxicity. The heightened expression of *Smn1* despite the unchanged *Casp3* expression implies its potential significance in the molecular and cellular responses activated during SCI and its involvement in regulating apoptosis-independent cell death mechanism. This conclusion highlights the need for further research to elucidate the specific molecular pathways influenced by elevated *Smn1* expression in the context of SCI, offering potential insights for targeted therapeutic interventions to enhance neuroprotection and promote recovery in the aftermath of spinal cord injuries.

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