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RESEARCH ARTICLE

The Effect of Nimodipine on Nuclear Factor Erythroid-2-Related Factor 2 (Nrf2) Activation in Neuron Cell Line SH-SY5Y Culture Exposed By Chronic Hyperglycemia

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Abstract

Introduction: Diabetes is a dysregulation of glucose metabolism due to defects in insulin secretion, decreased insulin sensitivity, or a combination of both that causes hyperglycemia. Diabetic neuropathy is induced by oxidative stress that results from excessive ROS production. When oxidative stress increases, it triggers a transcriptional response of antioxidant genes mediated by Nrf2. Based on the above mechanism, the researchers tried to use nimodipine as a calcium channel blocker which is neuroprotective to reduce Nrf2 activation. Objectives: This study aims to look at the effect of nimodipine on decreasing Nrf2 activation. Methods: This research was conducted in vitro using SH-SY5Y neuron cell culture induced hyperglycemia for 6 days with glucose concentrations of 5 mM, 25 mM, and 50 mM. Then the cell is harvested and given nimodipine for 30 minutes at a dose of 10 nM and 100 nM. Nrf2 expression was observed under a confocal microscope. Results: The results of nimodipine at doses of 10 nM and 100 nM were able to reduce the activation of Nrf2 in neuron cell culture that had been significantly induced by chronic hyperglycemia ($p < 0.05$) at 50 mM glucose concentration. Conclusion: It can be concluded that the administration of nimodipine doses of 10 nM and 100 nM can reduce Nrf2 activation in neuron cell cultures exposed to chronic hyperglycemia with glucose concentrations of 50 Mm.

Keyword: *Hyperglycemia, SH-SY5Y Cell, Nimodipine, Nrf2.*

Introduction

Diabetes is a dysregulation of glucose metabolism due to defects in insulin secretion, decreased insulin sensitivity, or a combination of both that will cause hyperglycemia. According to the International Diabetes Federation (IDF), the prevalence of diabetes mellitus worldwide in 2011 was 366 million, and by 2030 it is estimated that the prevalence will reach 552 million [1].

The International Diabetes Federation (IDF) also estimates that 1 in 11 adults aged 20-79 years of suffering from diabetes mellitus globally in 2015. This estimate is projected to increase to 642 million by 2040, and the largest increase will come from regions experiencing economic transition from low income to middle income [2]. Chronic hyperglycemia can cause damage to various body systems, especially nerves and blood vessels. Some of the consequences of diabetes that often occur are increased risk of heart disease and stroke, neuropathy (nerve damage) in the legs which increases the occurrence of foot ulcers, diabetic retinopathy, and kidney failure.

Most complications are neuropathy experienced by 54% of people with diabetes mellitus who are treated at the Hospital Dr. Cipto Mangunkusumo in 2011 was followed by diabetic retinopathy and proteinuria [3]. Diabetic neuropathy is a condition in which several dysfunctions occur due to diabetes mellitus.

Diabetic neuropathy is also the most common complication of diabetes mellitus [4]. Patients with diabetic neuropathy usually experience numbness, tingling, pain, and / or weakness that start from the feet and spreads to the proximal [5]. Diabetic neuropathy is induced by oxidative stress [6]. Oxidative stress results from excessive production of Reactive Oxygen Species (ROS) [7]. ROS also communicates with another pathway, namely calcium. The interaction between ROS and calcium can be considered as 2-way, where ROS can regulate calcium signaling, while calcium signaling is very important for ROS production.

Thus, increasing levels of Ca^{2+} activates ROS-producing enzymes and free radical formation [8]. Nuclear factor erythroid-2-related factor 2 (Nrf2) is a transcription factor that functions to regulate genes that encode antioxidants and detoxification enzymes [9]. Under hyperglycemia, oxidative stress and inflammation increase, triggering the transcriptional response of antioxidant genes mediated by Nrf2 [10]. Hyperglycemia Acute increases the expression of Nrf2, but persistent hyperglycemia decreases its expression and causes changes in various microvascular which will eventually lead to diabetic neuropathy [11].

So that, in the condition of diabetic neuropathy there is a change in the expression pattern of Nrf2 [12]. Based on the above mechanism, we try to use nimodipine to reduce Nrf2 levels in cells. Nimodipine is a dihydropyridine drug that inhibits the entry of calcium through L-type calcium channels [13]. Nimodipine can relatively penetrate the blood brain barrier properly, [14] and has a protective effect on neurotoxicity induced by OGD (oxygen-glucose deprivation) [15]. Therefore, this research studies aims to see the effect of nimodipine on the decrease in intracellular Nrf2 so that it can be an alternative in the treatment of diabetes.

Materials and Methods

This study uses a true experiment design in a laboratory in vitro. This study aims to determine the effect of calcium channel blocker (CCB) on intracellular concentration of Nrf2. This study uses SH-SY5Y cell line neurons that are cultured. The neuron culture was then exposed to the 5 mM, 25 mM, and 50 mM hyperglycemia conditions. Exposure to hyperglycemia was given for 6 days, then administered nimodipine 10 nM and 100 nM with a control group without nimodipine for 30 minutes. Then the Nrf2 parameter is checked using TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling). Nrf2 was examined using antibodies by immunoflurescence.

The analysis was compared between the hyperglycemia groups and nimodipine treatment. The research sample is the SH-SY5Y cell line neuron cell. This cell is then stored in a frozen form in a tube at -80^0C and put into a liquid nitrogen tank. To avoid multiple thawing, cells are stored in conical tubes in the form of aliquots. Determination of the size of the study sample is calculated using the formula of the sample size according to Federer: $(np - 1)(p - 1) \geq 16$ Where p is the number of treatments while n is the number of samples.

In this study the number of treatment groups was 4 as explained above so that based on calculations using the formula, a sample of 5 would be obtained, with 1 reserve, the total would be 6. The first procedure is thawing cells. The tools and materials used for cell thawing are laminar air flow (LAF), micropipette, blue tip, yellow tip, white tip, 10 cc syringe, filters, water baths, centrifuge, culture flask, conical tube, DMEM high glucose, penicillin- streptomycin 1%, L-Glutamine 200 mM 1%, FBS 10%.

Prepare a water bath by setting the temperature to 37^0C. Then with a micropipette for 12 ml of media consisting of FBS, L-Glutamine, penicillin-streptomycin, and high glucose DMEM. Homogenize all the ingredients above and take it with a 10 cc syringe and put it in a new conical tube using a filter. Put it in an incubator for about 15 minutes. Remove the cells from the freezer and thaw the cells in a previously prepared water bath. After melting, do pipetting on cells using a micropipette. Transfer the cells to the new conical tube and add the 5 ml media that has been made. Then centrifuge the cell at 1500 rpm for 3 minutes.

After that, dispose of the supernatant until the pellets remain. Add the media to the pellet as much as 2 ml and do pipetting. Insert the filtered media into 5 ml culture flasks. Then put 2 ml of pellets + media into the culture flask and do pipetting again. Then observe under the microscope. Then the cell passage is carried out with Laminar air

flow (LAF) tools, micropipets, blue tip, yellow tip, white tip, 10 cc syringe, filter, centrifuge, culture flask, conical tube, high glucose DMEM, 1% penicillin-streptomycin, L-Glutamine 200 mM 1%, FBS 10%, PBS 1%, accutase (EDTA). Make 20 ml of media then filter the media into a conical tube and place it in an incubator.

Flasks containing SH-SY5Y cells are removed from the incubator. The old media in the flask is sucked up using a 10 cc syringe and discarded. Then wash the cells 2x using PBS of 5 ml each. After washing put accutase into the flask and wait 2 minutes. After 2 minutes add the media to the flask to deactivate the accutase work. Then the contents of the flask are moved into the conical tube and centrifuge at 1500 rpm for 3 minutes.

When finished, remove the supernatant until the remaining pellets. Add 2 ml medium into the pellet and do pipetting to homogenize. Insert the media that has been made into 2 new flasks of 5 ml each and put the pellets into the 2 flasks. Then observe under the microscope. Third, platting was performed using laminar air flow (LAF), micropipette, blue tip, yellow tip, white tip, 10 cc syringe, filter, centrifuge, conical tube, well-24, DMEM high glucose, penicillin-streptomycin 1%, L-Glutamine 200 mM 1%, FBS 10%, PBS 1%, accutase (EDTA).

Make 15 ml of media, then filter the media into a conical tube and put the conical tube into incubator. Prepare well-24 and sterile cover glass. Insert the cover glass into well-24 each well 1 cover glass. After the well is filled with cover glass, insert media into each well of 300 µL each. Then the flask containing SH-SY5Y cells is removed from the incubator and discard the old media in the flask. Wash the flask using 2x PBS of 5 ml each and then remove the PBS. Add as much as 2 ml accutase and wait for 2 minutes.

After 2 minutes add 5 ml of media to the flask. Take all the contents of the flask and transfer to the conical tube. Do centrifuge at 1500 rpm for 3 minutes. After that, remove the supernatant until the remaining pellets. Add 2 ml medium into the pellet and do pipetting to homogenize. Insert pellets into wells of 40 µL wells while pipetting and observing under a microscope.

Hyperglycemia administration is done using Laminar air flow (LAF), micropipette, blue tip, yellow tip, white tip, 10 cc syringe, filter, conical tube, scales, spatula, high glucose DMEM, low glucose DMEM, low glucose penicillin-streptomycin 1%, L -Glutamine 200 mM 1%, FBS 10%, glucose. First weigh 0.018 g glucose, then make a 5 ml low glucose medium as much as 4 ml by mixing FBS, L-Glutamine, penicillin-streptomycin, and low glucose DMEM.

Then make 8 ml of high glucose medium as much as 8 ml by mixing FBS, L-Glutamine, penicillin-streptomycin, and high glucose DMEM. And make 50 ml of high glucose media as much as 4 ml by taking 4 ml of 25 mM glucose media then add to glucose that has been weighed before. The finished media is filtered into the conical tube according to its glucose concentration and then put in an incubator for approximately 30 minutes. After 30 minutes the media is removed and take the old media on well-24 using a syringe and then discarded.

With the micropipette, slowly insert the new media into each well of 300 µL each and observe under the microscope from day 0 to day 6. After that, add calcium channel blockers using laminar air flow (LAF), micropipette, blue tip, yellow tip, white tip, digital balance sheet, DMSO, culture media, and calcium channel blockers. First, calculate for stock nimodipine. Nimodipine is weighed using a digital balance sheet.

Then make nimodipine stock by dissolving nimodipine that has been weighed into 1 ml of DMSO. Make a stratified dilution to get nimodipine stock with molarities of 100 nM and 10 nM. After stratified dilution and adding to the media, remove well-24 and media that was previously made from the incubator. The well-24 label is suitable for the treatment to be given.

Take the media with a syringe and filter the media into a new conical tube according to the dose. Take the old media that is on well-24 and discard it. Enter the media slowly according to glucose concentration and the dose of nimodipine with a micropipette of 300 µL each. Then put well-24 into the incubator for 30 minutes. Perform Nrf2 staining using Laminar air flow (LAF), micropipette, blue tip, yellow tip, white tip, PBS 1%, Triton-x 0.1%, blocking buffer 5% (BSA in PBS), Ab primary Nrf2,

Ab secondary Nrf2; by washing using PBS 1% 3 times then permeabilitation with Triton-x 0.1% as much as 300 µL on each well for 10 minutes at room temperature. Then wash again using PBS 1% 3 times and given a 5% blocking buffer (BSA in PBS) for 30 minutes at room temperature. Take a blocking buffer and add a 1: 500 Ab Nrf2 primer (Primary Ab: blocking buffer) then put it into the 4^0C refrigerator overnight. After that remove the cell from the refrigerator and wait until the room temperature.

Wash with PBS 1% 3 times then add the secondary Ab Nrf2 1: 500 (secondary Ab: blocking buffer) and wait for 1 hour. Then wash it again using PBS 1% 3 times and observe using CLSM (Confocal Laser Scanning Microscope). After observing and obtaining Nrf2 activation data on neuron cells, data processing is performed.

Data analysis begins with the normality test and homogeneity test. Normality test of sample data for normal distribution is done by Shapiro-Wilk test, then homogeneity test is done by Homogeneity of Variance> 0.05. One Way Anova test was performed to test the mean data of more than two groups, and the correlation test. Saphiro Wilk and One Way Anova testing was carried out with SPSS for Windows 25.0 program.

Result and Discussion

Morphologically the results of observations of the influence of nimodipine on Nrf2 activation in neuron cell cultures exposed to hyperglycemia at various concentrations are shown in Figures 1, 2, and 3. Based on Figures 1, 2, and 3 there is a decrease in the activation of Nrf2 in SH-SY5Y neuron cell culture that has been induced by hyperglycemia. Besides appearing morphologically, the mean of Nrf2 activation also shows a decrease, which can be seen in figure 4. Whereas in table 1 shows the results of the One Way Anova test and the correlation test for each group.

The administration of nimodipine to SH-SY5Y neuron cell cultures exposed to chronic hyperglycemia with glucose concentrations of 5 mM and 50 mM showed statistically significant results, but there was no significant correlation in 5 mM glucose concentration. In the glucose group 5 mM + nimodipine 100 nM, glucose group 50 mM + nimodipine 10 nM, and glucose group 50 mM + nimodipine 100 nM there was a decrease in the mean activation of Nrf2.

Then at 25 mM glucose concentrations with 10 nM and 100 nM nimodipine doses also decreased Nrf2 activation visually and on average, but not statistically significant because the mean values of the 25 mM glucose group + nimodipine doses 10 nM and the 25 mM glucose group + nimodipine dose 100 nM approaches the mean value of the 25 mM glucose group without nimodipine.

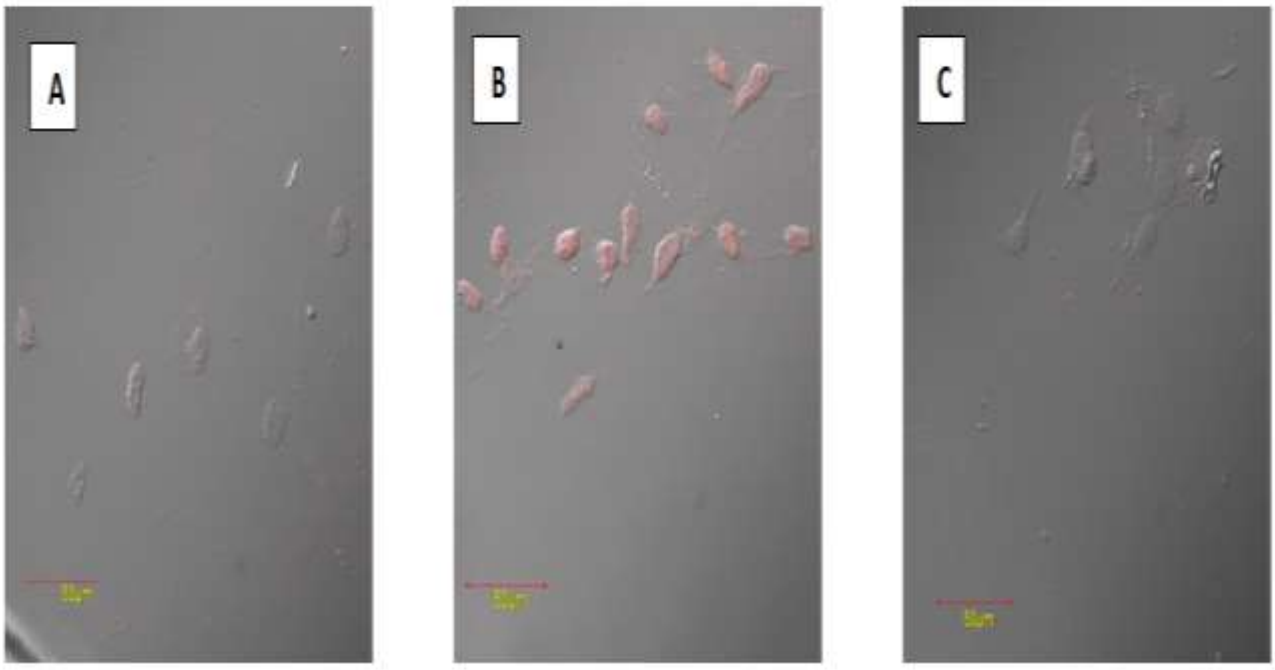

Figure 1: Activation of Nrf2 in SH-SY5Y cells with 5 mM hyperglycemia and nimodipine 0 nM (A), nimodipine 10 nM (B), nimodipine 100 nM (C) administration

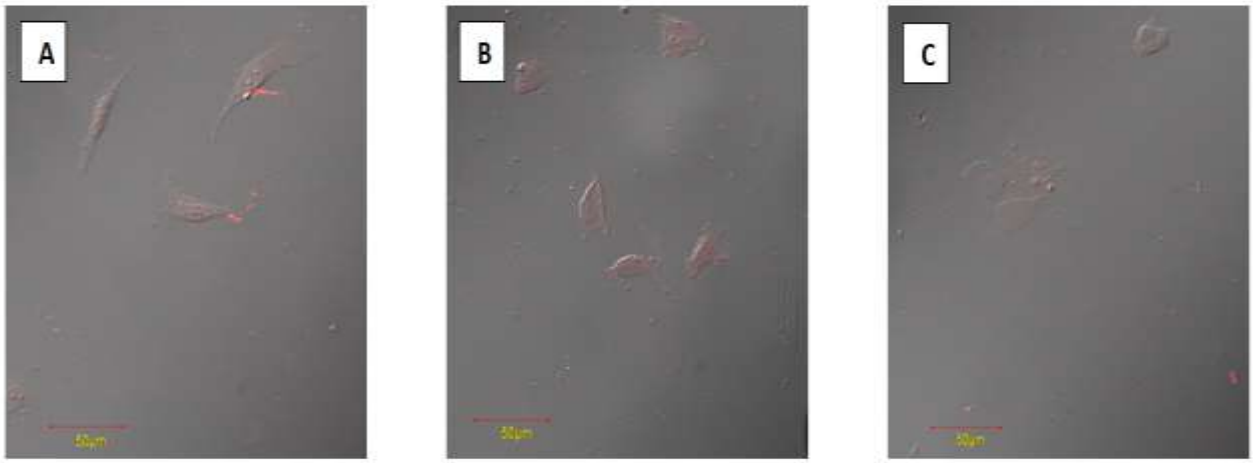

Figure 2: Activation of Nrf2 in SH-SY5Y cells with 25 mM hyperglycemia and nimodipine 0 nM (A), nimodipine 10 nM (B), nimodipine 100 nM (C) administration

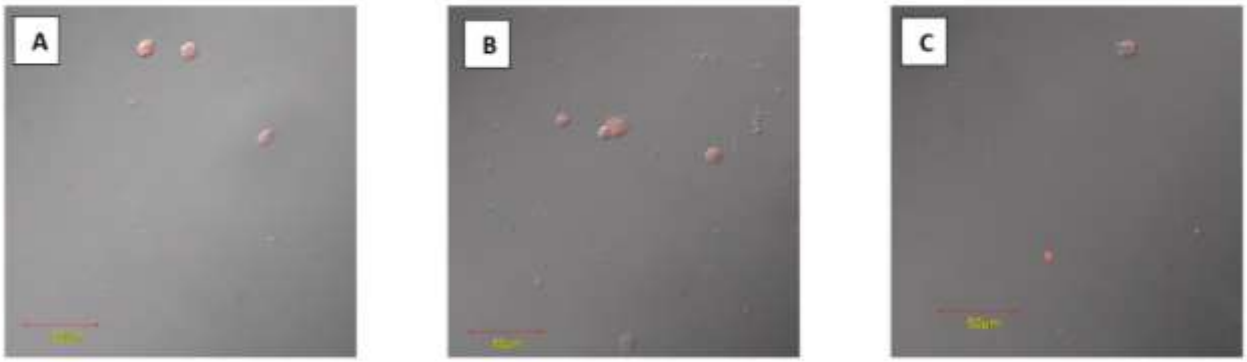

Figure 3: Activation of Nrf2 in SH-SY5Y cells with 50 mM hyperglycemia and nimodipine 0 nM (A), nimodipine 10 nM (B), nimodipine 100 nM (C) administration

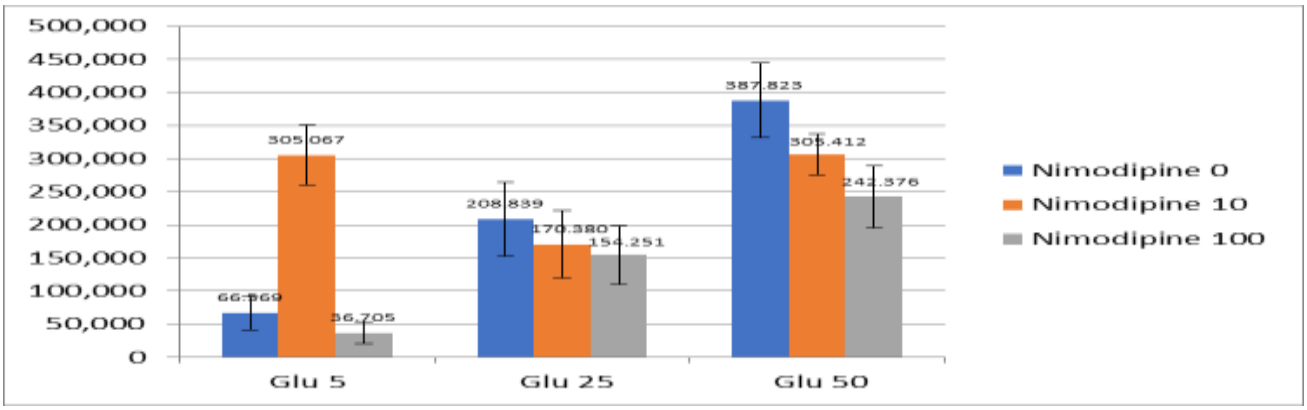

Figure 4: Mean effect of nimodipine on Nrf2 activation

Table 1: One Way Anova Test Results and Correlation Test

	One Way Anova	Correlation	
		Pearson	**Sig. (2- Tailed)**
Glucose 5 mM	0.000*	-0.099	0.760
Glucose 25 mM	0.336	-0.451	0.141
Glucose 50 mM	0.005*	-0.829	0.001*

*= Significant

This is consistent with research conducted by Schampel that nimodipine treatment can reduce levels of reactive oxygen species (ROS) in rat animals that have been induced by experimental autoimmune encephalomyelitis (EAE) before, and can also induce apoptosis and reduce the production of neurotoxic molecules such as mice. Nitric oxide and reactive oxygen species (ROS) both in vitro and in vivo [16].Research in Leisz also shows that nimodipine treatment can reduce cytotoxicity in Schwann cells against osmotic, oxidative, and heat stress significantly [17].

As previously explained elevated levels of reactive oxygen species (ROS) will trigger oxidative stress and induce a number of cell changes [18]. When oxidative stress occurs, Nrf2 is released from Keap1 and translocated to the nucleus, heterodimerization with one of the small Maf proteins. Then heterodimers recognize AREs, which are sequences in the regulatory region of the Nrf2 target gene, which are important for the recruitment of transcription factors [19].

From these statements it can be concluded that administration of nimodipine can decrease Nrf2 expression in SH-SY5Y neuron cell culture due to activation. the transcription factor Nrf2 itself is directly proportional to the level of reactive oxygen species (ROS) in cells [20]. In addition, nimodipine also has a neuroprotective effect apart from its vasodilator effect [21]. In a study conducted by Herzfeld, it showed that nimodipine significantly reduced ethanol-induced cell death by about 9% at all concentrations tested [22]. It was also said that nimodipine could produce a favorable environment for the regenerative process of neuron cells [16].

Whereas in the 5 mM glucose group by giving nimodipine at a dose of 10 mM there was an increase in Nrf2 activation, which was estimated because the 5 mM glucose concentration used was less than optimal. In a study conducted by Shi and Liu in 2006 stated that glucose levels are very important in regulating the redox state. The 25 mM glucose concentration is the optimal concentration for neurons maintaining the environment and shows the lowest ROS and cell death rates. Reducing glucose supply involves many pathological conditions such as stroke and contributes to systemic injury. This study shows that glucose levels have a large impact on ROS levels and cell viability in primary neurons with hypoxic conditions. At concentrations below the optimal concentration, it decreases cellular capacity (GSH / GSSG ratio) and increases cell-level ROS thereby causing cell damage to neurons in hypoxic conditions [23]. This is confirmed by research by Yoon and Oh which aims to find out whether changes in glucose concentrations in culture media can determine dopaminergic neuron cell death mode (DA) after 1-methyl-4-phenylpyridinium (MPP+) treatment, also mentioned that at a concentration of 5-10 mM glucose can cause cell death accompanied by DNA fragmentation and cell shrinkage [24].

Conclusion

Based on the results of research and discussion, it can be concluded that administration of nimodipine at doses of 10 nM and 100 nM can significantly reduce Nrf2 activation in SH-SY5Y neuron cell cultures exposed to chronic hyperglycemia with 50 mM glucose concentrations significantly. The effect of nimodipine on Nrf2 activation in SH-SY5Y neuron cell culture exposed to chronic hyperglycemia with 50 mM glucose concentration has a negative correlation, which means that the higher the dose of nimodipine given, the lower the activation of Nrf2 in neuron cell culture that has hyperglycemic exposure. It is necessary to research and review the optimal dose of nimodipine to reduce the activation of Nrf2 and various glucose concentrations that can be used for induction of hyperglycemia both in vivo and in vitro.

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