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RESEARCH ARTICLE

The Effect of Nimodipine to Reactive Oxygent Species (ROS) in SHSY-5Y Cell-Line Culture Expressed by Chronic Hyperglycemia

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Abstract

Background: Diabetes mellitus is a metabolic disease with a chronic hyperglycemia condition. Diabetic peripheral neuropathy is a condition that arises from long-term damage due to chronic and hyperglycemia combination of various factors and pathways. One of them is the occurrence of oxidative stress caused by an increase in ROS intracellular. The accumulation of ROS can activate other pathways and result in an ion imbalance Ca^{2+}. Nimodipine is a type L calcium channel blocker which has neuroprotective properties. Therefore, nimodipine is expected to reduce the expression of ROS in neurons that occur due to chronic hyperglycemia conditions. Objective: To determine the effect of nimodipine on ROS in culture of SH-SY5Y-induced cell-line neurons chronic hyperglycemia. Methods: Experimental research with in vitro methods using the exposed SH-SY5Y neuronal cell line chronic hyperglycemia. The study sample was divided into 9 groups, the control normoglycemic group, hyperglycemia control 25 mM and 50 mM, normoglycemia with nimodipine 10 nM and 100 nM, hyperglycemia 25 mM with nimodipine 10 nM and 100 nM, and 50 mM hyperglycemia group with nimodipine 10 nM and 100 nM. Nimodipine is given for 30 minutes on Sh-Sy5Y cells that have previously been exposed to hyperglycemia conditions chronic for 6 days. The intensity of intracellular ROS was observed by immunohistochemical methods on the sixth day. Results: In comparison of normoglycemic conditions (5 mM) and hyperglycemia 25 mM, normoglycemic conditions 5 mM resulting in a significantly higher ROS intensity ($p < 0.05$). Whereas in comparison normoglycemia (5 mM) and hyperglycemia 50 mM had a significant increase in ROS intensity ($p < 0.05$). Expose glucose 5 mM can increase ROS expression due to a series of processes due to hypoglycemic conditions. Exposure to high doses of glucose (50 mM) can increase ROS formation. Giving nimodipine to cells able to reduce the intensity of ROS on glucose exposure 5 mM, 25 mM and 50 mM significantly ($p < 0.05$). Conclusion: Chronic hyperglycemia conditions can increase the formation of ROS in SH-SY5Y and cell cultures nimodipine can significantly reduce ROS levels.

Keywords: *Nimodipine, SH-SY5Y, ROS, Hyperglycemia, Neuropathy.*

Introduction

Diabetes mellitus is a disease metabolic conditions with chronic hyperglycemia is a condition of increasing glucose levels in blood. According to RISKESDAS in 2013, the proportion of people with diabetes mellitus in Indonesia 6.9% for residents above the age of 15 years which is predicted to continue to increase [1].Conditions hyperglycemia is associated with change long-term microvascular exposure that can regarding body systems like on the retina, glomerulus, blood vessels, and peripheral nerves [2]. One important mechanism in the occurrence complication of peripheral nerve neuropathy is oxidative stress a result of an increase in ROS [3,4]. In hyperglycemia conditions, there will be an over process superoxide production in the electron transport chain coupled with low expression of enzymes antioxidants

such as catalase and glutathione peroxidase [5, 6]. Regulating calcium transport very sensitive to redox conditions will increase due to interference from the clearance system and release from calcium storage [7]. In addition, oxidative stress arising will trigger the activation of factors redoka transcriptions like NF-kB. Enhancement NF-kB activity is known to trigger it apoptosis [8].

Whereas NRF2 is capable inducing antioxidant transcription will decrease. As a result of this imbalance, will trigger neuroinflammation which can make damage to peripheral nerves [9].Nimodipine is a calcium channel blockers are known to have neuroprotective properties [10]. Further research is needed for know the role of nimodipine in reducing ROS expression in chronic hyperglycemia conditions.

Materials and Methods

Experimental Research

This research uses neuron culture the SH-SY5Y cell-line implemented at Central Laboratory of Life Sciences, University Brawijaya in July-August 2019. The purpose of this research is to find out the role of nimodipine in decreasing ROS expression on SH-SY5Y which has been induced chronic hyperglycemia. Cell exposed in hyperglycemia conditions for 6 days with glucose concentrations of 5 mM, 25 mM and 50 mM. Then given nimodipine 10 nM and 100 nM for 30 minutes. Observation of ROS intensity carried out by immunohistochemical methods and observed with a confocal microscope.

Sample

This SH-SY5Y neurona cell line is obtained from Cell Line Service, Germany. Grown in flask culture with growth media consisting from Dulbecco's Minimum Essential Medium (DMEM) high glucose supplemented with 10% Fetal Bovine Serum (FBS), 1% L-glutamine 200 mM, 1% Penucillin-Streptomicin. Then maintained in a 37°C incubator 5% CO_2, humidified water. Culture media are replaced every 3-7 days and observed cell growth. If growth reaches 80-90% confluence, then the cell is ready to be given treatment. This research was approved by UB Research Ethics Commission.

Induction of Hyperglycemia Conditions and Treatment Nimodipine

Hyperglycemia induction in SH-SY5Y cells is done by giving Dulbecco's Minimum Essential Medium (DMEM) purchased from Thermo fisher (11965092) with supplementation glucose. Glucose is dissolved first with DMSO to make stock solutions. Then prepared DMEM containing concentration glucose of 5 mM, 25 mM, and 50 mM Nimodipine which is used in the land of Santa Cruz Biotechnology, Dallas, Texas, USA (CAS 66085-59-4). SHSY5Y cells are plotted in a well culture dish inside there is already a glass cover.

Cells can hyperglycemia exposure if confluence of cells has reached> 60%. Cells that have been given exposure glucose was then incubated for 6 days with one time media replacement. After 6 days, group the treatment was added with nimodipine 10 nM and 100 nM to get 9 groups the sample. Cells that have been given nimodipine treatment it was then reincubated for 30 minute.

Reactive Oxygen Species (ROS)

H2DCF-DA to be used for Cell staining was purchased from Sigma-Aldrich, Israel (D6883 CAS 4091-99-0). Cells that have been given then treat it with H2DCF-DA for 60 minutes and fixed using 4% PFA. The intracellular ROS can then be observed with fluorescence (Ex/Em = 504/524 nm) for 30 minutes on the microplate reader. Observation result expressed in fluorescence emissions in one arbitrary unit (AU).

Data Analysis and Statistics

The intensity of the fluorescence that appears on observations then carried out the quantization process image using Olympus fluoview software Ver4.2a. Data obtained in the form of mean ± standard deviation. Data analysis is done by software SPSS statistics 24.0.

Testing using analysis One-Way ANOVA variations and correlation analysis with Pearson correlation. Data said to be significant statistically if we get p value <0.05.

Results

ROS expression in hyperglycemia conditions chronic and by administration of nimodipine in culture SH-SY5Y cell-line was observed on the sixth day using immunohistochemical methods. First of all carried out ter normality Komlogorov-Smirnov and Lavene homogeneity test obtained p> 0.05 in each sample group so the data can be said to be normally distributed and homogeneous. In the One-way ANOVA test obtained there is a significant increase in ROS in cells given glucose 5 mM and 50 mM compared cells with 25 mM glucose (p <0.001). At nimodipine 10 nM and 100 nM in each normoglycemic group and hyperglycemia found a decrease in expression Significant ROS (p <0.001).

In the correlation test Pearson found a positive correlation significant (p <0.001) between glucose administration and ROS concentration. Giving nimodipine to the group with glucose 5 mM, 25 mM, and 50 mM as well has a significant negative correlation (p <0.001). From the Pearson correlation test, it can be said the higher the dose of nimodipine given (dose 100 nM), the lower the excretion ROS that appears. Giving nimodipine 10 nM and 100 nM is able to effectively reduce ROS on chronic hyperglycemia conditions in the SHSY5Y cell-line culture. Immunohistochemical staining for observation of ROS in SH-SY5Y cells can be observed in the following image (Figure 1-4).

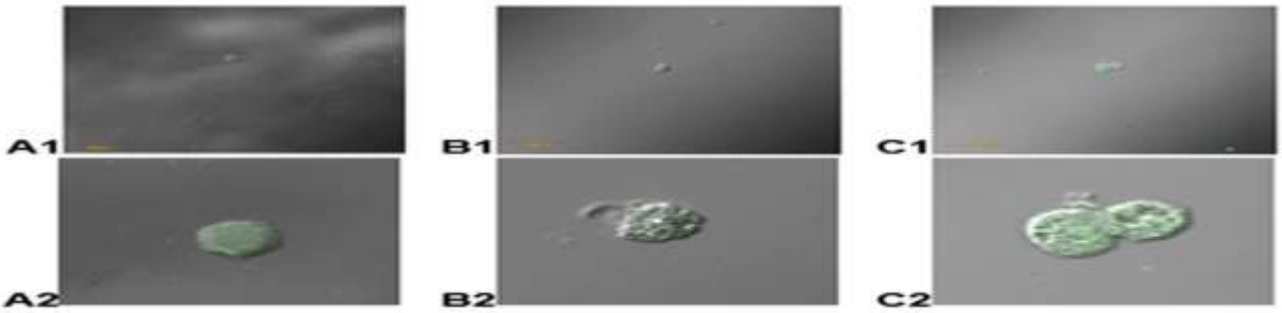

Figure 1: ROS concentration with A1-A2 glucose 5 mM, B1-B2 glucose 25 mM, C1-C2 glucose 50 mM. Cultured at 6th day, ROS staining with H_2DCFDA green colour, 400x magnification

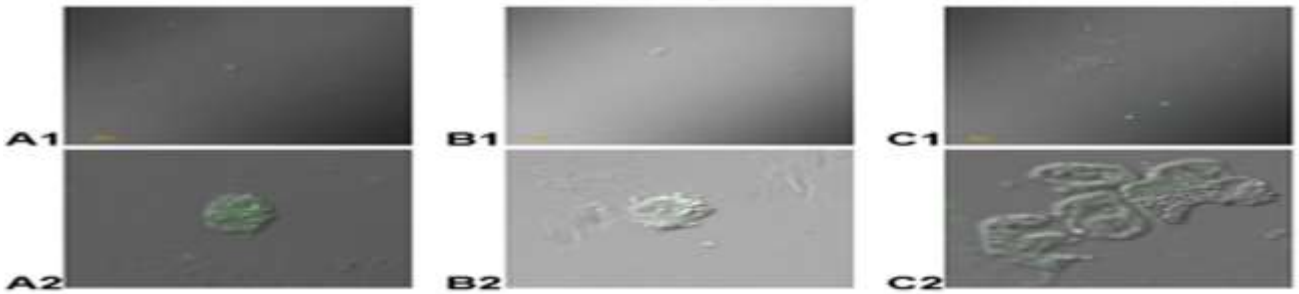

Figure 2: ROS concentration exposed with glucose 5 mM, A1-A2 with nimodipine 0 nM, B1-B2 with nimodipine 10 nM, C1-C2 nimodipine 100 nM. Cultured at 6th day, ROS staining with H_2DCFDA green colour, 400x magnification

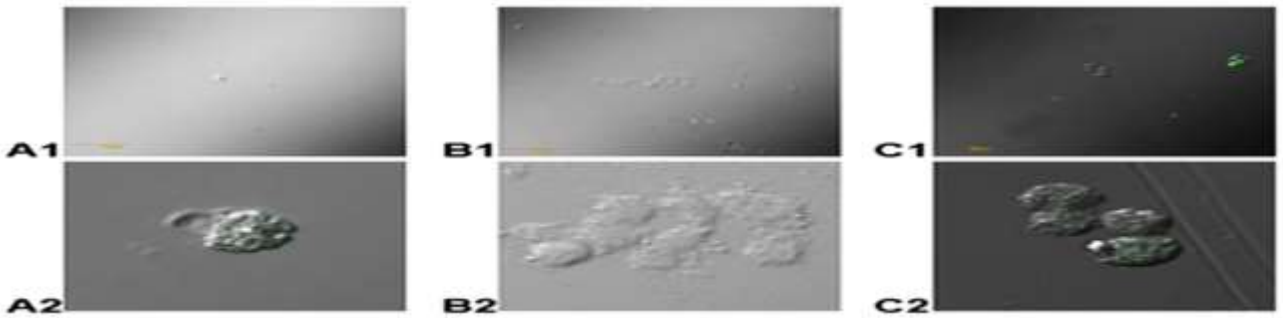

Figure 3: ROS concentration exposed with glucose 25 mM, A1-A2 with nimodipine 0 nM, B1-B2 with nimodipine 10 nM, C1-C2 nimodipine 100 nM. Cultured at 6th day, ROS staining with H_2DCFDA green colour, 400x magnification

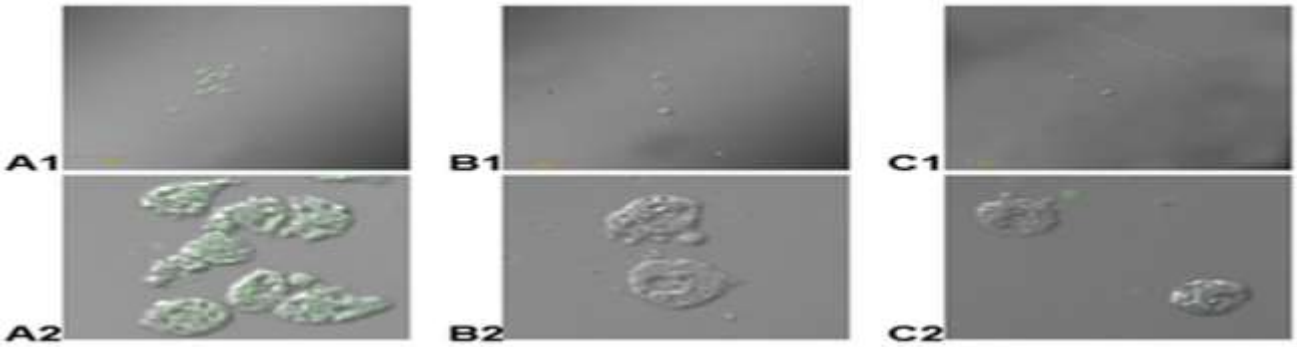

Figure 4: ROS concentration exposed with glucose 50 mM, A1-A2 with nimodipine 0 nM, B1-B2 with nimodipine 10 nM, C1-C2 nimodipine 100 nM. Cultured at 6th day, ROS staining with H_2DCFDA green colour, 400x magnification

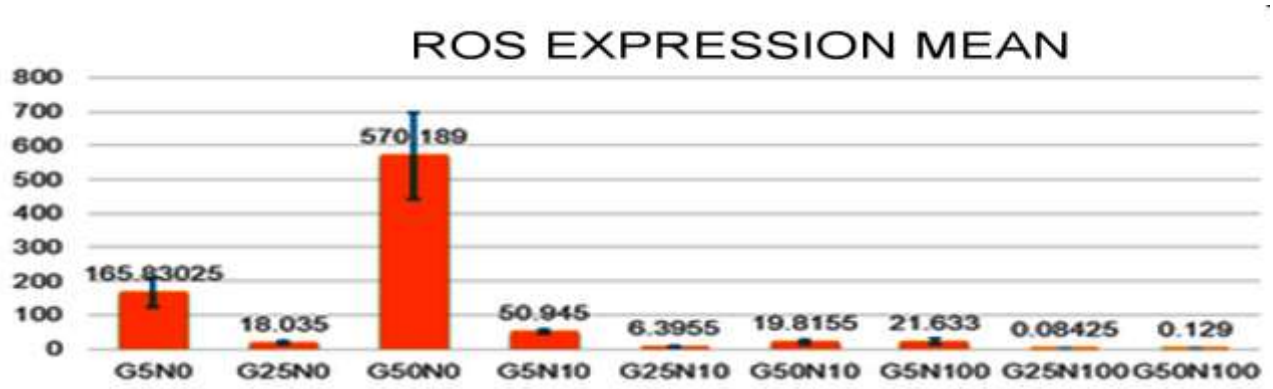

Figure 5 : ROS expression mean. Figure 5 shows ROS expression was highest in the hyperglycemia group with 50 mM glucose and the lowest ROS expression in hyperglycemia conditions with 25 mM glucose given 100 nM nimodipine

Discussion

Effect of Glucose on ROS

Visually, there are differences in fluorescent green fluorescent in cells exposed to chronic hyperglycemia conditions. Compared to cells exposed to glucose doses 25 mM, in cells exposed to glucose 50 mM for 6 days shows a glowing color. This matter indicates high intracellular ROS concentration.

Then, compared visually luminescent the color green between cells exposed to glucose 5 mM and 25 mM, in cells exposed to 5 mM glucose obtained luminous colors that are greener compared to cells exposed to 25 mM glucose. Accordingly statistically, a significant difference was found in the concentration of ROS exposed to glucose doses different. Statistically, administering doses glucose 5 mM and 50 mM increase ROS excretion significantly.

This is in accordance with research by Shi and Liu in 2006 conducted the research about the effects of glucose concentration on cortex neurons in mice. Shi and Liu found that dose glucose given will affect regulation redox status in these neurons states that the optimal glucose concentration is 25 mM which shows the ROS level and lowest cell death. In this research, it was concluded that for neuronal cells, glucose doses the most appropriate basal is 25 mM. On giving glucose whose concentration is lower than 25 mM, through an increased cell GSH / GSSG ratio and ROS production will also increase cause cell death.

For giving glucose whose concentration is more than 25 mM (25-100 mM) will increase ROS production and eventually also result in cell death. Previous studies on cerebral cortex newborn piglets, it has been known that during hypoglycemia an increase will occur peroxidation in the mitochondrial lipid membrane showed an increase in ROS production, although not explained further how detailed ROS generation process.

Increased production mitochondrial ROS when hypoglycemia is also capable interfering with mitochondrial function through the process lipid membrane peroxidase, oxidation of proteins mitochondria, which are components to the chain electron transport, and oxidation of DNA in the genome mitochondria.

Oxides caused by oxides on mitochondria are very closely related to decreased cytochrome oxidase activity, NADH dehydrogenase, and adenine nucleotide translocase [11].Previously explained that administering lower doses of glucose will increase the GSH / GSSG ratio and ROS production mitochondria. This is due to the efficacy of the system glutathione which is disrupted during hypoglycemic conditions.

For maintenance of the basal level of glutathione reduced in the brain requires glucose flow (flux) through jalus pentose phosphate to provide the reduction equivalent in the NADPH (nicotinamide adenine dinucleotide phosphate) form. Oxide lesions in the mitochondria due to the production of ROS mitochondria that increase during hypoglycaemia able to result in permanent changes in mitochondrial function in brain cells despite the condition has been returned to normoglycemia [12]. In high doses of glucose exposure, it will an increase in caspase activity and cytoplasmic.

There will also be a decrease in the number neurite / cell and neurite growth compared in normal glucose [13]. Cells activate its defense mechanism against exposure hyperglycemia. One of them is activation of responses antioxidant, one of the endogenous antioxidants for detoxification of ROS is SOD. Nrf2 in primary controls ARE, while ARE will regulate transcriptional activity for enzymes that are works as an endogenous antioxidant [14].

In liver cells, cardiomyoblasts and muscle cells plain, the presence of hyperglycemia conditions will induce mitochondrial fragmentation process. Although mitochondrial fragmentation is not direct influence the increase in ROS production, it is thought that this mitochondrial fragmentation will increase the surface area of the membrane so that take lots of substrates that will eventually increase ROS production in cells.

From various another process that occurs, the glucose uptake cycle increased and the ROS production process is induced by glucose, will cause cell death. High-dose glucose in human umbilical vein endothelial cells (HUVECs) cells can decrease hexokinase 2 (HK2) expression by suppressing expression HK2-transcription-factor peroxisome proliferator activated receptor gamma (PPARγ) finally can decrease the expression of Bcl-2 (B-cell lymphoma 2) will weaken mitochondrial Voltage-dependent anion-selective channel 1 (VDAC-1) interactions with HK2 and Bcl-2 resulting strengthening of Bax (apoptosis regulator) bond to VDAC1. Strengthening ties this will increase membrane permeability mitochondria thus inducing cell apoptosis [15].

The hypothesis in this study is the upregulation of HK2 or PPARγ can reduce apoptosis vascular endothelial cells and prevent complications vascular due to diabetes. In this study used glucose 5.5, 16.5 or 33 mM for 3 days and then results in decreased cell viability, increased cell apoptosis and cleaved-caspase-3 levels on exposure to high doses of glucose (16.5 and 33 mM). This study also showed glucose exposure high doses are able to induce apoptosis through weakening of the structure and function of the mitochondria) [16]. With thus, according to this study that exposure glucose in high doses with a length of treatment 6 days can increase the concentration of ROS significant.

Effect of Nimodipine on ROS on Exposure Chronic Hyperglycemia

In statistical analysis, it can be concluded nimodipine 10 nM and 100 nM apparently can reduce the concentration of ROS on SH-SY5Y cell culture. On induction with glucose 5 mM and 25 mM giving nimodipine 10 nM and 100 nM, makes a significant difference.

On glucose induction of 50 mM, administration of nimodipine to cell culture was significant with respect to ROS concentration; however there is no meaningful difference between administration of nimodipine in doses of 10 nM and 100 nM. Visually, it can be seen that at administration of nimodipine, green glow which appears less and less. More and more reduced green color in the picture shows the lower the concentration of ROS in cells.

Concluded that in this study, giving nimodipine can reduce ROS in neuron culture SH-SY5Y cell-line. In the study of Herzfel and his friends in 2014 about the

Neuro2a cells given a different stress stimulus and then given nimodipine, stating that nimodipine can improve neuroprotection function with prevent Ca^{2+} overload. The presence of intracellular Ca^{2+} excess, glutamate excitotoxic activity and ROS production plays a role in neuronal cell death [17].Although the mechanism of neuroprotective properties nimodipine is still unknown. On the model this research, administration of nimodipine will increase survival of cells and prevent neuronal cell death due to stress induced by various kinds of mechanisms.

Cell survival increases significantly when cells are treated with nimodipine before application of stress to Neuro2a cells nimodipine is an example dihydropyridine-L-calcium-channel blocker class. Another example of this type of drug is amlodipine. Research by Hirooka et al 2008, in his experiments on stress relations oxidative with sympathetic inhibitory effect in mice with the hypertension model concluded that giving amlodipine as L-type CCB can reduce oxidative stress in areas of the brain rat [18].

In Vitro research using SH-SY5Y cells added with U-373 astrocytic cells and human monocytic cell line THP-1 to find out its cytotoxicity by Hashioka et al. In astrocyte cells and the microglia were given nimodipine of 10 μM and verapamil by 30 μM later exposed to stimulants in the form of interferon gamma (IFNγ). Then it was concluded that CCB administration, especially nimodipine, can be a useful therapeutic option and can be used in diseases degenerative diseases, such as Alzheimer's [19]. ROS has been proven to have a relationship directly with Ca^{2+} homeostasis in cells and other intracellular organelles.

Calcium canal contained in the endoplasmic reticulum and mitochondria can doubled intrinsically with an increase in ROS. Although the relationship between voltages gated calcium channel (VGCC) with ROS is still unknown clearly, but the expression of type L calcium channels down-regulation can occur because of the process inflammation. The reason is because of the calcium response resulting in depolarition of the membrane and voltage gated calcium channels opening. In stressful conditions oxidative, an increase in calcium due mobilization from extracellular and from storage intracellular in the endoplasmic reticulum due to influx Abnormal calcium observed in muscle cells smooth muscle, resulting in a decrease in the amount of protein L type Ca^{2+} channel but there is no change functional characteristics [20,21].

As a result of depletion of calcium storage in endoplasmic reticulum can result activation of calcium influx capacist resulting in extended calcium elevation. Reduction of the functional amount of calcium channels occurs because of the long-term adaptation that results SERCA mechanism (sarco-endoplasmic reticulm Ca^{2+} ATPase) blockers that decrease storage of calcium in the endoplasmic reticulum. SERCA plays a role in calcium leaking pathways from endoplasmic reticulum [22].

There is a decrease in number the functional calcium channels above that are related with apoptosis and the term oxidative stress process long, thus can be suspected of being the reason for giving 100 mg nimodipine to cells exposed to chronic hyperglycemia at a dose of 50 mM did not make a significant change compared with administration of nimodipine 10 nM.

Conclusion

Chronic hyperglycemia condition capable increase the formation of ROS in SHSY5Y cell cultures and nimodipine can reduce ROS levels significantly. In this study stated that nimodipine is able to be an alternative therapy for diabetes neuropathy because it has properties neuroprotective.

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References

1. Depkes RI (2013) Riset Kesehatan Dasar. Jakarta: Badan Penelitian dan

pengembangan Kesehatan Kementrian Kesehatan RI.

2. Larsen RP, Kronenberg H, Melmed S, Polonsky K (2015) Williams Textbook of Endocrinology 13th edition. Philadelphia: Elsevier.
3. Veves A, Malik RA (2007) Diabetic Neuropathy Clinical Management. Totowa, New Jersey: Humana Press.
4. Chopra M, Kaul K, Tarr J, Choudhury R, Kohner E, Chibber R (2012) Oxidative stress in diabetic neuropathy: source of reactive oxygen species. Endocrinology Studies, 2(2), e6.
5. Premkumar LS, Pabbidi RM (2013) Diabetic peripheral neuropathy: Role of reactive oxygen and nitrogen species. Cell Biochem Biophys, 67:373-383.
6. Kaneto H, Katakami N, Matsuhisa M, Matsuoka TA (2010) Role of reactive oxygen species in the progression of type 2diabetes and atherosclerosis. Mediators of inflammation, 453-892.
7. Camello-Almaraz C, Gomez-Pinilla PJ, Pozo MJ, Camello PJ (2006) Mitochondrial reactive oxygen species and Ca^{2+} signaling. AJP: Cell Physiology, 291(5), C1082–C1088.
8. Kaltschmidt B, Kaltschmidt C (2009) NFkappaB in the nervous system. Cold Spring Harbor perspectives in biology, 1(3): a001-271.
9. Liu XF, Hao JL, Xie T, Malik TH, Lu CB, Liu C, Zhou DD (2017) Nrf2 as a target for prevention of age-related and diabetic cataracts by against oxidative stress. Aging Cell, 16(5): 934-942.
10. Shi H, Liu KJ (2006) Effects of glucose concentration on redox status in rat primary cortical neurons under hypoxia. Neuroscience letters, 410(1): 57-61.
11. Nohl H, Gille L, Staniek K (2005) Intracellular generation of reactive oxygen species by mitochondria, 69: 719-723.
12. Mcgowan JE, Chen L, Gao D, Trush M, Wei C (2006) Increased mitochondrial reactive oxygen species production in newborn brain during hypoglycemia, 399, 111-114.
13. Chopra M, Kaul K, Tarr J, Choudhury R, Kohner E, Chibber R (2012) Oxidative stress in diabetic neuropathy: source of reactive oxygen species. Endocrinology Studies, 2(2): e6.
14. Vincent AM, Kato K, Mclean LL, Soules ME, Feldman EL, Al VET (2009) Sensory neurons and Schwann cells respond to oxidative stress by increasing antioxidant defense mechanisms. Antioxid Redox Signal, 11(3):425-38.
15. Grefte S (2015) Interactions between mitochondrial reactive oxygen species and cellular glucose metabolism. Archives of Toxicology, 89(8): 1209-1226.
16. Zhang J, Guo Y, Ge W, Zhou X, Pan M (2019) High glucose induces apoptosis of HUVECs in a mitochondria-dependent manner by suppressing hexokinase 2 expression. Experimental and therapeutic medicine, 18(1): 621-629.
17. Herzfeld E, Strauss C, Simmermacher S, Bork K (2014) Investigation of the neuroprotective impact of nimodipine on neuro2a cells by means of a surgery-like stress model. Int. J. Mol. Sci., 15(10): 18453-65.
18. Hirooka Y, Kimura Y, Nozoe M, Sagara Y (2006) Amlodipine-induced reduction of oxidative stress in the brain is associated with sympatho-inhibitory effects in stroke-prone spontaneously hypertensive rats. Hypertens Res, 29(1): 49-56.
19. Hashioka S, Klegeris A, McGeer PL (2012) Inhibition of human astrocyte and microglia neurotoxicity by calcium channel blockers. Neuropharmacology, 63(4): 685-691.
20. Lam A, Karekar P, Shah K, Hariharan G, Fleyshman M, Kaur H, Rao SG (2018) Drosophila voltage-gated calcium channel α 1-subunits regulate cardiac function in the aging heart. Scientific Reports, 1-13.
21. Ca L (2001) Down-regulation of L-type calcium channels in inflamed circular smooth muscle cells of the canine colon. Gastroenterology, 120(2):480-9.
22. Pinton P, Giorgi C, Siviero R, Zecchini E, Rizzuto R (2008) Calcium and apoptosis: ER mitochondria Ca^{2+} transfer in the control of apoptosis. Oncogene, 27(50): 6407-6418.

