Available online at www.ijpcr.com
International Journal of Pharmaceutical and Clinical Research 2017; 9(2): 114-117

DOI number: 10.25258/ijpcr.v9i1.8292

ISSN- 0975 1556

Research Article

Neuroprotection of 17β-Estradiol Against D-Glucose Exposure Effect on Tyrosine Hydroxylase Expression and Apoptotic Cells in Zebrafish (*Danio rerio*) Brain During Early Development

Kartika Rahma[1], Mitsuyo Kishida[2*], Sasangka Prasetyawan[1], Aulanni'am Aulanni'am[1,3*]

[1]*Biochemistry Laboratory, Department of Chemistry, Faculty of Sciences, Brawijaya University, Malang, Indonesia*
[2]*New Frontier Science Department, Graduate School of Science and Technology, Kumamoto University, Kumamoto, Japan*
[3]*Faculty of Veterinary Medicine, Brawijaya University, Malang, Indonesia*

Available Online: 25th February, 2017

ABSTRACT

Impaired glucose balance affects the regeneration of neurotransmitters in the brain. One of neurotransmitters that is known to have an important role in the brain is dopamine. Dopamine production by dopaminergic (DA) neurons is regulated by the enzyme tyrosine hydroxylase (TH). Previous research has been reported that a decline on TH expression in DA neurons was a result of exposure to excess glucose. One contributing factor is an excess amount of glucose, which interfere homeostasis in the body by producing reactive oxygen species (ROS) more and cause oxidative stress. This condition can cause damage to cells in the brain, including DA neurons, to cell death occurs (apoptosis). The characteristic of neuroprotective owned by the hormone estrogen allegedly able to protect nerve cells from the damaging effects of this. The aim of this study is to explore the involvement of 17β-Estradiol as neuroprotection on the effects of glucose exposure during early development on dopaminergic neurons. This study used zebrafish (*Danio rerio*) embryos as animal model. They were divided into 4 groups: negative control group, hyperglycemia group, group that was co-incubated glucose and estrogen, and the group that only incubated with estrogen alone. Cell death (apoptosis) in the brain was observed using acridine orange staining and TH expression was observed using immunocytochemistry. The results showed that the embryos were exposed to 5% glucose significantly ($p < 0.05$) increased the number of apoptotic cells and decreased the expression of TH. While the results of the analysis of embryos in co-incubation group with estrogen showed improvement with decreased apoptotic cells and the increasing expression of TH up to the normal amount. It can be concluded that estrogen shown to have neuroprotective effect in protecting the decreasing TH expression, as the effects of glucose exposure, which could potentially lead to diabetes, by acting as an antioxidant through cellular mechanisms in the central nervous system.

Keywords: Estrogen, Glucose, Tyrosine hydroxylase, Apoptotic cell.

INTRODUCTION

Hyperglycemia cause metabolic disorder in the body including lowering brain function. Many studies have shown that the activity of neurons in the brain is affected by glucose[1]. Energy from glucose metabolism in the brain is used for the differentiation of neurons and neurotransmitters biosynthesis[2]. One of the neurotransmitter that has an important role in regulating cognitive and motor function is dopamine. Production of dopamine by DA neurons is regulated by tyrosine hydroxylase (TH). This enzyme catalyzes the formation of L-DOPA from tyrosine, which will be transformed into dopamine after through the decarboxylation process. A previous study reported that zebrafish embryos exposed to high concentrations of glucose could lower the expression of TH. One cause of the degeneration of neurons in the brain is the accumulation of ROS. In the circumstances disproportionate amount of ROS with antioxidants, oxidative stress causes damage to neuronal death[3]. Dopaminergic neurons in the brain to deteriorate due to increased production of reactive oxygen species (ROS) under high glucose levels[4]. This condition causes damage to cells in the brain, including in DA neurons resulting cell death (apoptosis).

Estrogen is known to have a role in the regulation of tyrosine hydroxylase (TH) in dopaminergic (DA) neurons. Recently, estrogen is known to have other important effects in the nervous system as neuroprotection. Many of the findings approved the experimental results showed that estrogen has a neuroprotective effect[5]. However, the action of estrogen as neuoprotection on DA neurons in animal model of diabetes exposed to high concentrations of glucose has not been widely studied.

This study was studied using zebrafish (*Danio rerio*) as an animal model that has high regeneration of neuron and genetic similarity to mammals, particularly humans. This research studied neuroprotective action of estrogen

**Author for Correspondence: aulani@ub.ac.id*

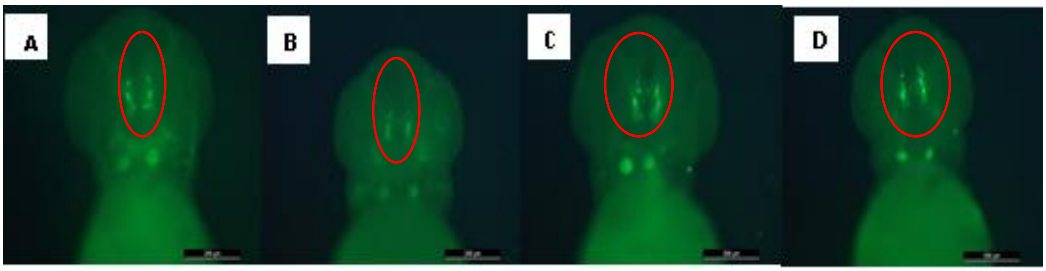

Figure 1: Tyrosine hydroxylase (TH) expression in zebrafish embryos (48 hpf) brain (midbrain) by immunocytochemistry technique.

Description: A. Negative control embryos; B. Embryos were incubated in 5% glucose for 24 hours; C. Embryos were co-incubated in 5% glucose and 0.0001µM 17β-estradiol for 24 hours; D. Embryos were incubated in 0.0001µM 17β-estradiol for 24 hours.: TH expression

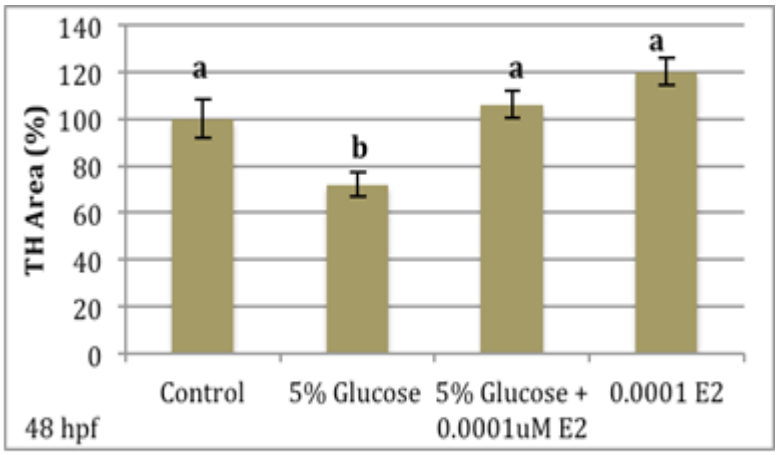

Figure 2: Percentage of Total Area of TH Expression at 48 hpf Zebrafish Embryonic Brain using ICC-TH technique.

protecting the DA neurons in the brain, due to glucose (5%) exposure by the co-incubation of glucose with 0.0001µM 17β-estradiol during early embryonic development of zebrafish (*Danio rerio*). Furthermore, the observation on the level of apoptosis and TH enzyme expression was conducted since it is the key or as rate limiting step of the series of DA neuron activity in the brain in the early development of zebrafish (*Danio* rerio) embryos.

MATERIALS AND METHODS

Fish maintenance

Adult zebrafish (*Danio rorio*) were maintained at 28-30 ºC in recirculating system (aquatic habitats) and kept in a cycle of 14 hours light; 10 hours dark. The embryos of zebrafish were collected and raised in embryo medium (EM). After 24 hours post-fertilization, the collected embryos were mechanically dechorionated to optimize transdermal drug delivery of glucose exposure.

Chemical Treatments and Exposure

D-Glucose was obtained from Sigma-Aldrich, embryos were divided into 4 groups: control, D-glucose exposure, Co-incubation with D-glucose and estrogen, and estrogen exposure. The dose of D-glucose used was 5% and dose of estrogen used was 0.0001µM dissolved in 0.01% DMSO.

Immunocytochemistry Tyrosine Hydroxylase (TH)

Bleached embryos aged 48 hours post-fertilization (hpf) were incubated in dH_2O for 60 minutes and replaced with 100% acetone for 8 minutes incubation at -20 ºC. After all incubations should be treated with tri-fold washed of PBSTX. Apply a blocking solution of 1% NGS in 3% BSA-PBS to the treated embryos for 3 hours at room temperature (RT) and then washed with PBSTX 2-times per hour. Incubate embryos in 1% NGS/PBS containing the primary antibody at 4 ºC, overnight. The primary antibody TH used was at 1:1000 from 1:10 stock of TH antibody. The next morning, embryos were washed in PBSTX for 4 hours (one-hour/wash) at room temperature. Add the anti-mouse secondary antibody 1:500 alexafluor488 and incubate at 4 ºC for 2 hours. After incubation, wash in PBSTX at least three times (5-min/wash), then the stained embryos were observed in 0.5% agarose gel under fluorescence microscope using GFP3 filter. The resulting photos were analyzed using ImageJ 1.47v software.

Acridine Orange Staining

Embryos were incubated in 0.003% 1-phenyl-2-thiourea (PTU) to inhibit pigment formation for 4 days post-fertilization (dpf). Apoptotic staining used was 5µg/mL acridine orange in EM for 45 minutes in incubator and dark. Then stained embryos were washed three times in EM, then anesthetized in 0.016% tricaine to ease the observation. The stained embryos were observed under fluorescence microscope using GFP3 filter. The resulting photos were analyzed using ImageJ 1.47v software.

Statistical Analysis

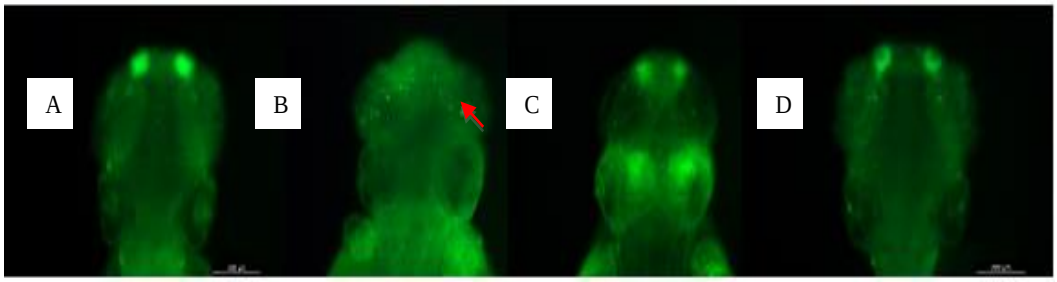

Figure 3: Expression of apoptotic cells on zebrafish embryos (4 dpf) by using acridine orange staining. Description: A. Negative control embryos; B. Embryos were incubated in 5% glucose for 3 days; C. Embryos were co-incubated in 5% glucose and 0.0001µM 17β-estradiol for 3 days; D. Embryos were incubated in 0.0001µM 17β-estradiol for 3 days. → : this arrow pointing towards the apoptotic cell.

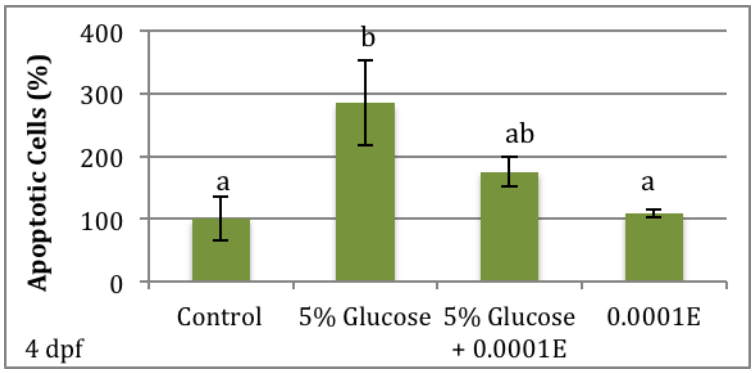

Figure 4: Percentage Apoptotic cell by using acridine orange after on Zebrafish Embryos brain at 4 dpf.

Data are expressed as mean ± the standard error of the mean (SEM). For quantitative analysis of multiple comparisons using a one-way ANOVA followed by post-hoc's test for normal data or Kruskal-Wallis test followed by Mann-Whitney test due to the abnormal and inhomogeneous data. A *p*-value less than 0.05 considered statistically significant. Statistical analysis was performed using SPSS version 16 software.

RESULTS AND DISCUSSION

Tyrosine Hydroxylase Expression

Based on these data, it is clear that exposure to excess glucose in the early embryonic development has a negative impact on the enzyme of dopamine production. Incubation for 24 hours at 24 hpf embryos in dose of 5% glucose has probably sufficient cause hyperglycemia, followed by insulin resistance and glucose dysregulation causing degeneration of DA neurons activity[6]. Not only disruption of the hormone insulin, hyperglycemia environment can trigger nerve cell damage as a result of increased production of ROS[4]. The accumulation of ROS in the brain would also interfere the activity of DA neurons. It can also explain the decrease on TH expression in the brains of embryonic zebrafish (*Danio rerio*) which TH role is the key to the whole process in DA neurons by acting as catalyst.

In this case, to prove the neuroprotective action of estrogen in protecting the effects of high glucose concentration exposure, the embryos was co-incubated in 5% glucose and 0.0001µM 17β-estradiol. The study confirmed that the decreasing TH expressed in midbrain (Fig.1) of zebrafish embryos given 5% glucose was significantly rescued by estrogen (0.0001µM) co-incubation. The TH expression of co-incubated group was equal to normal TH expression in control embryos (Fig. 2). Thus, besides estrogen has been known to be able to regulate dopamine production, it may have neuroprotective action by protecting the decreasing of TH expression, caused by glucose exposure which could potentially lead to diabetes, through cellular mechanisms in the central nervous system.

Levels of Apoptotic Cells

Many studies established high glucose concentration due to diabetes increases apoptosis especially in brain and retina[7]. Previous result showed 0.0001µM 17β-estradiol rescued the effect of glucose exposure on TH expression. Hence, to investigate whether estrogen also has protective action on apoptotic cells induced by glucose, co-incubation glucose (5%) with 0.0001µM estrogen was performed on embryos at 24 hpf. Acridine orange staining was used to define the brain cell apoptosis in each group at 96 hpf. Stained apoptotic cells in brain only incubated in 5% glucose were increased compared with brain of control group (Fig. 3). The cause apoptosis can be varied, one of them due to the activation of p53 because of DNA damage[8]. In the hyperglycemic state, glucose metabolism could trigger oxidative stress through glycolysis mechanisms. Some pathways that produce ROS through this are polyols, hexsosamine, PKC, and the formation of AGE[9]. The accumulation of ROS initiate to oxidative stress in the body causes damage and death (apoptosis) of neurons[10].

Co-incubation of glucose with estrogen prevents cell death in brain due to glucose effect after 3 days co-

incubation. Statistical test results embryos were co-incubated in 5% glucose and 0.0001μM estrogen showed partial decrease compared to group incubated in glucose alone (Fig. 4). This showed that the effect of neuroprotection provided by 17β-estradiol exposure against the effects of 5% glucose on zebrafish embryos brain by acting as an antioxidant in cells at central nervous system to suppress the occurrence of apoptosis in nerve cells exposed to glucose. As antioxidant, estrogen may counteract free radical by scavenging mechanism. Thus, it prevents the activation of apoptotic pathways in neuronal cells

CONCLUSION

The co-incubation of 0.0001μM 17β-estradiol as neuroprotection is able increased expression of tyrosine hydroxylase (TH) and decreased the apoptotic brain cells during early development of zebrafish (*Danio rerio*) embryos caused by D-glucose exposure.

ACKNOWLEDGEMENT

The author would like to thank the Japan Student Services Organization (JASSO) for financial support of this research.

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