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Original Research Article

## The Effect of Increased Glucose Induction on GSH Levels in Insulin Gaussia Luciferase (iGL) Cells Derived from Rat Pancreatic Beta Cells

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### Abstract

**Background:** Prolonged hyperglycaemia can make the pancreatic beta cells work harder and cause fatigue. When this happens, it can trigger oxidative stress reactions, which can produce free radical compounds that can damage pancreatic beta cells. The body compensates by activating protective mechanisms such as the production of antioxidant compounds to reduce the levels of free radicals in the cells. One such compound is glutathione (GSH). Insulin Gaussia Luciferase (iGL) cells are a cell line derived from rat pancreatic beta cells. These cells can be used as a model of oxidative stress in hyperglycaemia to measure GSH levels and there are no studies using iGL cells to measure GSH levels. Therefore, in this study, the iGL cells are used as the object of research. The reason for using serial plasma GSH measurements is to determine gradual differences in changes in GSH levels and to provide variations and new data for further GSH research.

**Objective:** To investigate the effect of GSH levels on glucose toxicity condition through in vitro experiments on iGL cells.

**Methods:** The study used 5 different glucose concentrations of 11, 16.5, 22, 33, and 44 mM with the addition of iGL cell growth medium exposed for 7 days. We measured the amount of intracellular GSH using a colourimetric method (MBS, 2540412) at a wavelength of 405 nm with microplate reader (AMR-100 Allsheng). The analysis used in this study was a one-way ANOVA test. Differences between groups were tested using SPSS.

**Results:** The results of this study showed that there was an increasing trend in total GSH levels on the third and seventh day. with the average increase on third day being 20.16 nmol and the average increase on seventh day being 19.58 nmol.

**Conclusion:** In this study it can be concluded that there was a trend of increasing GSH/cell levels on the first day of observation because the cells were trying to maintain their homeostasis in hyperglycemia conditions. on the third and seventh days the cells experienced a decrease which was thought to be due to glucotoxicity, so that giving high glucose levels had an effect on decreasing GSH.

**Keywords:** Colorimetric method; GSH; Hyperglycaemia; iGL cells; Oxidative stress

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### INTRODUCTION

In everyday life, people have a high risk of being exposed to glucose through the consumption of foods with a high glucose content. This makes people susceptible to hyperglycaemia (high blood sugar). When this happens, the body tries to lower blood glucose levels by releasing insulin from the beta cells of the pancreas. Excess glucose in the blood, which continues to rise, can

cause the beta cells to work very hard and lead to fatigue. So, it can cause dysfunction in the beta cells of the pancreas.<sup>10</sup>

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When this condition occurs continuously, it induces oxidative stress reactions that can produce free radicals through glucose autooxidation and protein glycosylation.<sup>10</sup> Reactive oxygen species (ROS) have the potential to cause dysfunction in affected organs through cell damage and lead to cell apoptosis.<sup>11</sup> In addition to beta-cell fatigue, chronic oxidative stress can cause dysfunction in affected organs. Oxidative stress is also one of the factors leading to decreased intracellular production of insulin biosynthesis due to suppression of pancreatic-duodenal homeobox-1 (PDX-1) gene expression.<sup>6</sup> Thus, the amount of production secreted into the extracellular space is also decreased.<sup>4</sup> The human body can fall into the condition of diabetes mellitus. According to the International Diabetes Federation (IDF), in 2019 there will be an estimated 463 million people aged 20-79 years with a prevalence of 9.3% of the total population of the same age.<sup>2,5</sup> It is estimated that with increasing age, the prevalence will increase by 19.9% or 111.2 million people aged 65-79 years, and it is even estimated that by 2030 it will be 578 million people. In the South-East Asian region, Indonesia is the third most affected country with a prevalence of 11.3%. Diabetes is the most common chronic disease causing death. It can lead to microvascular and macrovascular complications.<sup>15,20</sup>

Therefore, the body compensates by activating the body's defence mechanisms by producing antioxidants. One of these antioxidants is glutathione (GSH), which has the function of maintaining the balance between pro-oxidants and anti-oxidants.<sup>8</sup> In this study, insulin gausia luciferase (iGL) cells were used. These cells are a subset of cells from the beta cells of the rat pancreas.<sup>9,16</sup> iGL cells have the properties of the Gaussia luciferase protein, which acts as a measure of insulin secretion in a fluorescent state and can be visualised in 2 and 3 dimensions.<sup>16</sup> In addition, these cells can be used to model oxidative stress conditions when exposed to high glucose. This is the reason why we used this cell. It is still very rare for this cell to be used in research, so the data from this research can be used as a reference for further research. The reason for using serial plasma GSH measurements is to determine gradual differences in changes in GSH levels and to provide variations and new data for further GSH research.

## MATERIALS AND METHODS

### 1. Cell culture

The cells used are Insulin Gaussia Luciferase (iGL) cells derived from rat pancreatic beta cells (Cosmo Bio Co., 2020).<sup>3,9</sup> iGL cells are cultured in RPMI 1640 medium incubated at 37°C with 5% CO<sub>2</sub>. RPMI 1640 (Gibco) consists of L-glutamine, phenol red and HEPES. 18 Additives were added in the form of 5% FBS, 1 mM pyruvic acid, 500 µM monothiolglycerol and 200 µg/mL G-148. Before culturing the cells in the medium, the cells were thawed in a water bath for two minutes. Then 1 mL of medium was added to the cryotube. The cells were immediately transferred to a 15 mL tube (Corning, 430791 USA) and 10 mL of medium was added. The cells were centrifuged at 300 G for five minutes and the supernatant discarded until cell pellets were visible. 1 mL of media suspension was prepared

from the cells and the number of cells was counted using an automated cell counter (LUNA Automated Cell Counter, Logos Biosystem, South Korea). 8.5 x 10<sup>5</sup> cells were plated in a 100 x 20 mm Petri dish (Corning, 430167 USA). The cultured cells should be replaced on the third or fourth day. Cells that have reached 80-90% confluence can be passaged.

The first thing that has to be done in the cell passaging process is the submersion of the cell media. Cells were rinsed with sterile PBS (Gibco, 181912014 USA). Cells were treated with 0.05% trypsin-EDTA (Gibco, 25300054 USA) at 1:10 and incubated at 37°C for two minutes. Immediately after incubation, 10 mL of medium was added to deactivate the trypsin. Cells removed from the plate are transferred to a 15 mL tube, the cells are centrifuged and the supernatant discarded. Cells used as suspensions were counted using an automatic cell counter. Cells of 8.5 x 10<sup>5</sup> were resuspended in new 100 x 20 mm petridishes.

### 2. Addition of glucose to the culture medium

At this stage, the treatment process was carried out by adding a glucose solution to the RPMI 1640 medium. The RPMI 1640 medium was used as the control medium because it contained 11 mM glucose. The glucose solutions to be prepared were 16.5 mM, 22 mM, 33 mM and 44 mM. The glucose (Sigma, 24895335 USA) required is 19.8, 79.2, 118.2 and 39.6 mg by weighing on an analytical balance. The glucose which has been weighed is then dissolved in 15 ml of medium in a 15 ml tube (Corning, 430829 USA).

### 3. Colorimetric method for measuring GSH levels

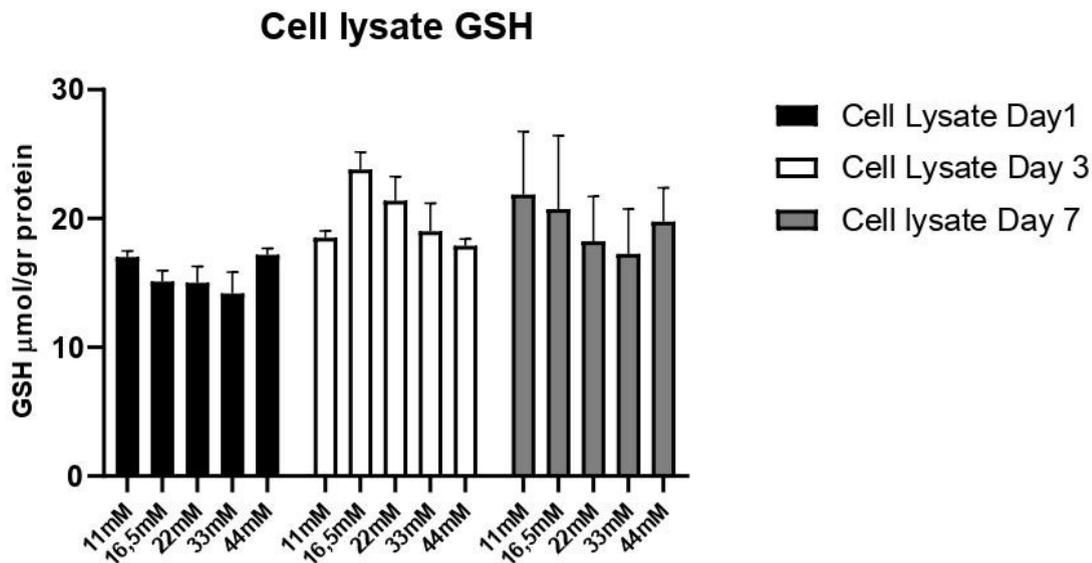
The GSH Colorimetric Assay Kit (MBS, 2540412) was used to measure GSH production. A microplate reader with a wavelength of 405 nm is used for this method. The harvested iGL cells were then rinsed with 0.1 M PBS (Gibco, 181912014 USA); pH 7.4 as much as 0.3 to 0.5 mL. Cells were disrupted by sonicating or grinding by hand in an ice-water bath. Take 0,1 mL of the disrupted cells and add 0,1 mL of Reagent 1 solution. Centrifuge the cells for ten minutes and collect the supernatant for measurement. Prepare the standard solution by dissolving 1 mmol/L GSH standard solution in GSH standard diluent. Standards are prepared in series and then added to the standard wells. Add the supernatant to be measured to the sample well. Allow the evenly mixed wells to stand for five minutes at room temperature. The results of the OD values are plotted on the standard curve and used to calculate the GSH levels.<sup>8</sup>

### Analysis of Statics

Each experiment was performed in triplicate. Software used to analyse normally distributed data, expressed as the standard deviation of the mean (version 19.0; IBM, Chicago, IL, USA). One-way ANOVA analysis was used for group comparisons. A statistically significant difference was defined as  $p < 0.05$ .

**Table 1.** iGL/GSH cell data for each day of observation

Concentration	Days 1			Days 3			Days 7		
	cells	GSH total (μmol)	GSH/C cells (nmol)	cells	GSH total (μmol)	GSH/C cells (nmol)	cells	GSH total (μmol)	GSH/C cells (nmol)
11 mM	257.333	17	0,066	468.000	18,5	0,039	1.553.333	21,9	0,014
16,5 mM	125.000	15,1	0,12	508.333	23,8	0,046	2.000.000	20,7	0,010
22 mM	59.767	15	0,25	391.667	21,4	0,054	956.000	18,2	0,019
33 mM	41.800	14,2	0,339	232.000	19	0,081	625.667	17,3	0,027
44 mM	41.800	17,2	0,411	139.000	17,9	0,128	1.103.333	19,8	0,017

**Figure 1.** iGL cell lysate GSH level at various state of high glucose induction (44, 33, 22, 16,5 and 11mM as control) related times to incubation (1, 3, and 7 days)

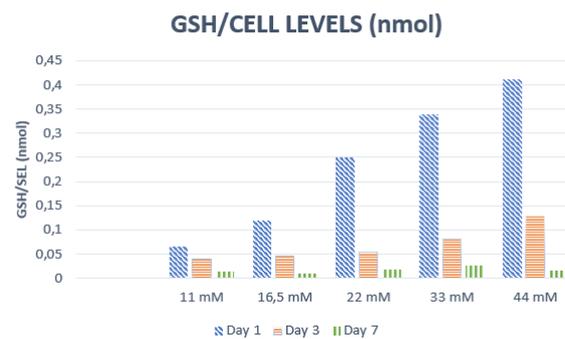
## RESULTS

### Data of total GSH on iGL lysate cells at each concentration

The results of measuring total GSH levels by the colourimetric method showed that there were no significant differences between the groups on a daily basis ( $p > 0.05$ ). These results are shown in Figure 1. However, these data show that there is an increasing trend on the third and seventh day. Table 1 is a description of the value of the results from Figure 1 and Figure 2, where in Figure 2 is the result of the conversion from μmol to nmol.

### Data of GSH/Cell on iGL lysate cells at each concentration

The GSH/Cell value was also obtained from a more detailed analysis by comparing the number of cells with the total GSH content for each concentration. It was then observed in Figure 2 that there was an increasing trend in GSH/Cell levels on the first day when high concentrations of glucose were administered. Then, there was a decreasing trend in GSH/Cell levels each day after exposure to high glucose concentrations for seven days.

**Figure 2.** Graph of Cell Lysate GSH/Cells Measurements in iGL Cells by Comparing the Same on Different Days

## DISCUSSION

Observations on the first day showed that iGL cells were trying to maintain homeostasis by increasing GSH levels. The response of beta cells to acute changes in extracellular glucose is to secrete insulin. High levels of extracellular glucose will increase glucose metabolism in cells. As a result, levels of reactive oxygen species (ROS) increase, advanced glycosylation (AGEs), activation of protein kinase C (PKC), also increase, polyol pathway activity also increases. Hexosamine metabolism and angiotensin II

production are also increased. Hyperglycemia stimulates insulin biosynthesis up to 50-fold in pancreatic beta cells. During this process, three disulfide bonds are formed per insulin molecule, causing the release of millions of ROS molecules per minute or free radicals to increase.

This is because the increase in ROS levels in the cell stimulates the activation of Nrf2 transcription. The Nrf2 protein is a regulator that plays a role in cell defence against oxidants.<sup>12</sup> Nrf2 will regulate the basal and induced expression of a number of genes that play a role in the antioxidant response. ROS will oxidise cysteine, then Nrf2 will phosphorylate serine 40 (ser40) with the help of PKC and then translocate to the nucleus. In this state, Nrf2 forms a complex with the small protein Maf and binds to the antioxidant response element (ARE) located in the regulatory region of the Nrf2 target gene.<sup>1</sup>

Nrf2 regulates more than 100 different genes involved in oxidative stress and cell survival. One of these functions is to control the expression of genes responsible for replenishing the cytosolic pool of NADPH, which is used as a reducing agent to maintain and regenerate cellular detoxification systems and antioxidant defences such as glutathione.<sup>4</sup> The regulated gene expression is glucose-6-phosphate dehydrogenase from the pentose phosphate pathway (hexose monophosphate shunt). One of the main products produced by this pathway is NADPH (nicotinamide adenine dinucleotide phosphate), which is a reduced form of NADPH.<sup>13</sup> Then NADPH is used as a cofactor by glutathione reductase to reduce oxidized glutathione (GSSG → 2 GSH).<sup>13,19</sup> After that, the reduced GSH will react with ROS compounds and GSH will be oxidized again to GSSG to reduce intracellular ROS levels, this recycling cycle depends on NADPH.

There is increasing evidence that activation of the Nrf2 pathway during hyperglycaemia and ROS generation is essential for the protection and maintenance of functional β (beta) cell mass. Nrf2 will promote macro-autophagy as an additional β (beta) cell defence mechanism by increasing β(beta) cell proliferation. When iGL cells were further exposed to high concentrations of glucose, there was a downward trend because iGL cells had entered a state of glucotoxicity.<sup>7</sup> This is evidenced by a decrease in antioxidant capacity in hyperglycaemia. A decrease in antioxidant levels can stimulate apoptosis in beta cells through the intrinsic pathway. This stress signal can lead to the activation of the proapoptotic proteins BAK and BAX, which are part of the BCL-2 protein family.<sup>17</sup>

These two proteins mediate mitochondrial outer membrane permeability by forming pores in the mitochondrial outer membrane, and oxidative stress can increase the opening of the mitochondrial permeability transition (mPTP) pores. Prolonged exposure to mPTP can induce cell death by increasing oxidative stress leading to ATP depletion and/or by inducing matrix swelling and subsequent rupture of the mitochondrial outer membrane. Apoptotic molecules such as apoptosis inducing factor (AIF), SMAC/DIABLO or cytochrome c are released during the permeabilization process.<sup>1</sup>

## CONCLUSION

This study concluded that administering high glucose concentrations over a period of 7 days resulted in an increasing trend in GSH/cell levels on the first day of observation. This condition is to maintain homeostasis in acute hyperglycemia. On days 3 to 7 of observation, GSH/cell levels decreased. This condition is thought to be due to glucotoxicity occurring due to a decrease in antioxidants. Giving high glucose has an effect on reducing GSH levels.

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