

## Hyperglycemia may induce a decrease in myoblast viability: A Preliminary Study



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### ABSTRACT

**Background:** Hyperglycemia plays a significant role in developing insulin resistance, leading to a continuous decline in metabolism. Uncontrolled long-term hyperglycemia has deleterious effects on muscles, leading to a decline in muscle mass, impaired capillarization, and muscle atrophy. We identified the effect of hyperglycemia on reducing myoblast cell viability through observation using the MTT assay.

**Methods:** Primary culture of myoblast cells was performed using rat's muscle tissue which was further induced by hyperglycemia by adding D-glucose with concentrations of 10 mM, 25 mM, and 50 mM in the plating media. Cell viability was observed using the MTT assay by identifying the absorbance using an ELISA reader with  $\lambda=550-600$  nm (595 nm).

**Results:** There was a significant decrease in cell viability ( $p=0.000$ ) due to administering various glucose concentrations in the plating media. There is a negative correlation between glucose concentration and myoblast cell viability ( $P\text{-value} = 0.000$ ;  $t = -8.284$ ;  $r = -0.926$ ).

**Conclusion:** Elevated blood sugar levels result in a significant reduction in the viability of myoblast cells, as indicated by the MTT assay. The MTT assay proved invaluable for precisely evaluating myoblast cell viability in the context of hyperglycemia.

**Keywords:** Cell Toxicity, Hyperglycemia, MTT Assay, Myoblast Cell Viability.

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### INTRODUCTION

The Centers for Disease Control and Prevention has declared that about 30.5 million Americans have diabetes and about 84 million with prediabetes; this number is expected to continue to increase over the next several decades. Increasing obesity, the aging population, and decreased physical activity are one of the reasons for the increased incidence of hyperglycemia.<sup>1</sup> Based on Indonesian Ministry of Health data in 2018, there are around 10 million people with diabetes in Indonesia, which is expected to increase to 30 million in 2030. Diabetes can cause complications such as high blood pressure, kidney failure, several lower limb amputations, and heart attacks.<sup>2</sup> According to the American Diabetes Association Guidelines, blood glucose levels between 100–126 mg/dL (5.6 to 7 mmol/L) are hyperglycemia. Meanwhile, blood glucose levels greater than 7 mmol/L are considered diabetes.<sup>3</sup>

Hyperglycemia contributes to the progressive occurrence of insulin resistance, triggering a continuous

decline in metabolism. A mild increase in blood glucose concentration (25–50 mg/dL) in healthy individuals can induce insulin retention within 72 hours due to the accumulation of triglycerides in the skeletal muscles. Uncontrolled long-term hyperglycemia can cause a decrease in muscle mass, muscle capillarization, and muscle atrophy.<sup>3,4</sup> Hyperglycemia can produce reactive oxygen species (ROS) in several body tissues, causing disturbances in the function of the heart (cardiomyopathy), lungs (vasculopathy), kidneys (nephropathy), eyes (retinopathy), nerves (neuropathy), and muscles (atrophy). ROS at certain levels can also act as signaling molecules to activate a stress response that is beneficial to the body.<sup>5</sup>

To determine the effect of glucose concentration on myoblast cell viability in vitro, it is necessary to carry out laboratory tests using the tetrazolium salt method. The MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) has a lipophilic structure so that it can pass through the mitochondrial cell

membrane.<sup>6,7</sup> The colorimetric MTT assay was first introduced by Mosmann in 1983 to identify metabolic activity in living cells and is a suitable method to be applied in an in-vitro study.<sup>8</sup> The reduction reaction in the MTT assay results from the breakdown of the yellow tetrazolium salt into purplish formazan crystals, which are used as a reference in detecting cell proliferation. Proliferating cell mitochondria will absorb the MTT reagent and turn purple due to the formation of formazan.<sup>9,10</sup> Besides being used as a viability test in cell culture; the MTT assay is also widely used to determine the ability of cell proliferation and also to test the cytotoxicity of a compound against cells. This method is among the most popular due to its simple and time-saving procedure.<sup>7</sup>

Our research aims to determine the effect of glucose in various concentrations on the viability of myoblast cell cultures using the MTT assay method. We supplemented the glucose to the medium with different concentrations, namely 10 mM, 25 mM, and 50 mM.

## MATERIAL AND METHODS

### Ethics

This research has been approved by the Ethical Committee of Research in Medical Health, Faculty of Medicine, Public Health, and Nursing of Universitas Gadjah Mada, Yogyakarta, Indonesia (Reference number: KE/FK/0600/EC).

### Animals

Adult male Sprague-Dawley rats, aged 12 weeks and weight 250-300 gr, were obtained from the Faculty of Pharmacy, Universitas Gadjah Mada. Rats were kept in a standard room with 19-23°C and 12-hour light-dark cycle. Animal sampling was carried out randomly on rats that did not have gastrocnemius muscle anatomy abnormalities as inclusion criteria. Primary cultures of myoblast cells contaminated with fungi were excluded from this study.

### Isolation and primary culture of myoblast cell

A sampling of muscle tissue explants and isolation, harvesting, and subculture of myoblast cells based on the modified protocol from Vaughan and Lamia (2019).<sup>11</sup> This procedure has been established and adopted in other studies of myoblast culture, one of them in a study conducted by Kihara Y et al. through examination of Pax7 expression as a biomarker of myoblast cells.<sup>12</sup> We modified the method without adding an amniotic fluid medium supplement because the component and function are almost the same as fetal bovine serum (FBS), while our research has used FBS with high concentration.<sup>13</sup>

This research using plating media which contains 25.5% Dulbecco's modified Eagle's medium (DMEM; Gibco), 40% fetal bovine serum (FBS; Sigma US Origin), 25.5% HAM'S F12 (Gibco, #11765054-500mL), 2% penicillin-streptomycin (Sigma, #P4333-100mL) and 1% Fungizone (FZ; Amphotericin B solution, Sigma #A2942-20 mL). Rats were anesthetized using Ketamine HCL 10 mg/kg BW intramuscularly. The surgical area on the lower leg (gastrocnemius muscle) is cleaned by shaving and disinfected with alcohol. A 3 cm long incision was performed on the lower leg using a sterile minor surgical tool to

remove the gastrocnemius muscle. We put the gastrocnemius muscle tissue into a 15 mL conical tube containing 8 mL PBS on ice with a temperature of 8-15°C and transported it to the class II biological safety cabinet (BSC). The conical tube containing the gastrocnemius muscle tissue was transferred to a new conical tube containing 5 mL PBS for tissue cleaning. The tissue was cleaned by inverting the conical tube slowly for 1 minute and then transferred to a new conical for repeated washing. The tissue was then transferred to a new petri dish for a final washing using 5 mL PBS.

The tissue was cut into small sizes ( $\pm 1$  mm) in a new petri dish containing 0.5 mL of plating media. The tissue explant pieces were then transferred to a tissue culture dish and stored in a 37°C, 5% CO<sub>2</sub> incubator. Tissue attachment was observed 24 hours after the culture was performed using an inverted microscope. Observation of cell culture and replacement of plating media was carried out every 48 hours until the cells were 80-90% confluence, harvested after the third passage, then subjected to hyperglycemia induction treatment using D-glucose in concentrations of 10 mM, 25 mM, and 50 mM.

### Hyperglycemia induction

This study used a plating media containing 5.5 mM D-glucose concentration as a control group. In the hyperglycemic group, a certain amount of D-Glucose (Merck #1083371000) was added to the medium at concentrations of 10 mM, 25 mM, and 50 mM in each group for 48 hours.<sup>14-16</sup>

### Cell viability detection using MTT assay

The MTT assay was carried out according to the guidelines from the Cancer Chemoprevention Research Center (CCRC), Faculty of Pharmacy, Universitas Gadjah Mada.<sup>17</sup> Myoblast cell growth was observed using an inverted microscope. 80% confluent cells were then prepared for induction of hyperglycemia using D-glucose at concentrations of 10 mM, 25 mM, and 50 mM on a plating medium in 96-well plates to a final 100  $\mu$ L/well volume. Cell distribution was observed using an inverted microscope and incubated in a CO<sub>2</sub> incubator for 48 hours. After 48 hours of incubation, the plate was taken

from the incubator, and the cell media was discarded by turning the plate 180° over the waste bin at a 15 cm distance. An amount of 100  $\mu$ L PBS was added to the well plate for washing and discarded by turning the plate 180° (same as the previous step). Each well was added with 100  $\mu$ L MTT reagent and incubated the cells for 4 hours in a CO<sub>2</sub> incubator (37°C) until formazan was formed. Observation of the cell condition was done using an inverted microscope. An amount of 100  $\mu$ L DMSO stopper was added to each well when the formazan was formed, and the absorbance was recorded using an ELISA reader with  $\lambda=550-600$  nm (595 nm).

### Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) version 26.0 for Windows. The MTT assay data distribution test was conducted using the Shapiro-Wilk, followed by the One-way ANOVA test. Post-hoc test was performed using LSD and continued with a linear regression test, which aims to determine the linear relationship between glucose concentration and myoblast cell viability. The correlation coefficient (r) was carried out using the Pearson correlation test to determine the direction and strength of the correlation between glucose concentration and myoblast cell viability.

## RESULTS

Myoblast cells began to be observed to grow within 48 hours (day-2) after the tissue explant culture on the tissue culture dish, as shown in [Figure 1](#). Cells began to show confluency on the 14th day. Statistical analysis showed a significant decrease in myoblast cell viability due to the administration of D-glucose in culture media at concentrations of 10 mM, 25 mM, and 50 mM compared to the control group, as seen in [Table 1](#).

The results of the linear regression test showed a P-value = 0.000 with a t = -8.284. These results indicate a negative relationship between glucose concentration and myoblast cell viability. It can be concluded that the higher the glucose concentration in the culture medium, the lower the viability of myoblast cells.

**Table 1.** Cell viability in control dan hyperglycemia-induced groups

Group	Mean $\pm$ SD	One-way ANOVA (P-value)	Linear regression		Correlation coefficient (r)
			t	P-value	
Control	0.427 $\pm$ 0.007 <sup>a</sup>				
10 mM	0.382 $\pm$ 0.014 <sup>b</sup>	0.000*	-8.284	0.000**	-0.926***
25 mM	0.376 $\pm$ 0.014 <sup>c</sup>				
50 mM	0.341 $\pm$ 0.006 <sup>d</sup>				

\*P-value = 0.000 showed the significant difference between groups tested by One-way ANOVA;

\*\*P-value = 0.000; t = -8.284 showed a negative correlation between glucose concentration and myoblast cell viability;

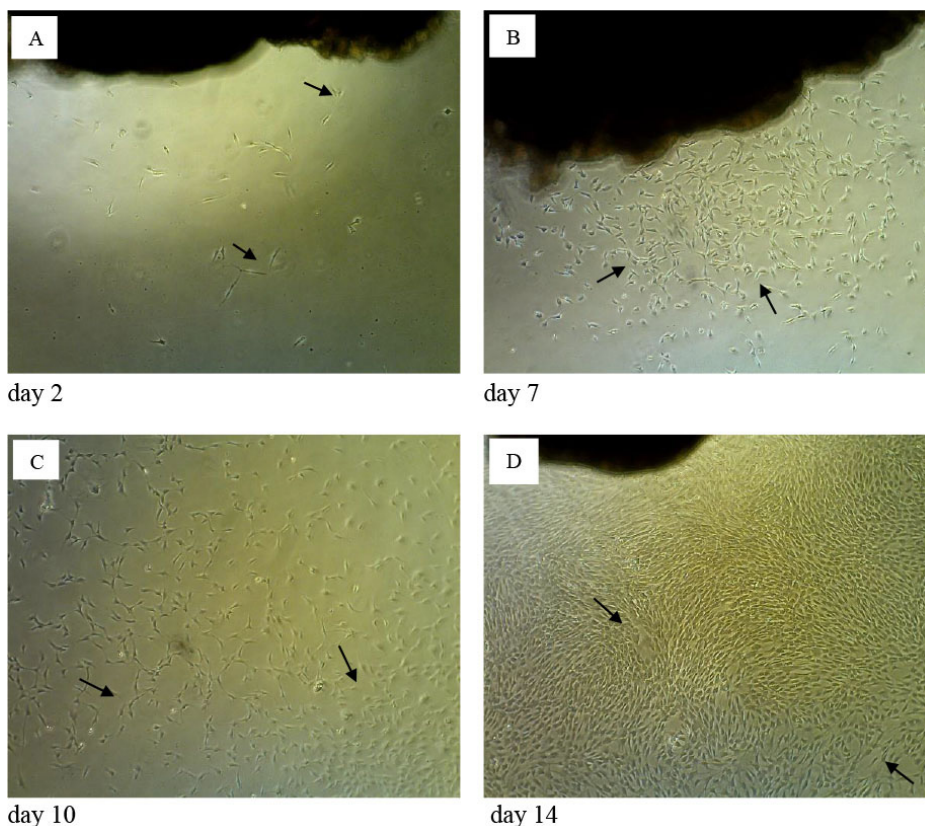
\*\*\*r = -0.926 showed a strong negative correlation between glucose concentration and myoblast cell viability;

a: Cell viability in the 10 mM, 25 mM, and 50 mM groups was significantly lower than in the control group;

b: Cell viability in the 10 mM group was significantly lower than in the control group and significantly higher than in the 50 mM group;

c: Cell viability in the 25 mM group was significantly lower than in the control and 10 mM groups and significantly higher than in the 50 mM group;

d: Cell viability in the 50 mM group was significantly lower than in the control, 10 mM, and 25 mM groups.



**Figure 1.** Myoblast cell culture growth.

A: Myoblast cells start growing from tissue explants on day 2; B: Several cells spread around the explant tissue on the 7th day; C: cells are expanding all over the surface of the tissue culture dish on the 10th day; D: cells begin to confluent on day 14.

## DISCUSSION

This study successfully showed that glucose administration in concentrations of 10 mM, 25 mM, and 50 mM reduced myoblast cell viability in vitro through observation using the MTT assay method. The cell-based assay method is frequently

conducted to assess the compound accumulation to determine the impact of the substances on cell growth and their toxicity effect that leads to cell damage or cell death. These assays are widely utilized to assess receptor binding and various signal transduction events that may encompass genetic reporter expression,

cellular trafficking, and organelle function. Several assay methods exist to estimate the number of viable eukaryotic cells, particularly in multi-well formats where data is captured using a plate reader. The methods described include tetrazolium reduction (MTT, MTS, XTT, and WST-1 assay), resazurin reduction, protease markers, and ATP detection.<sup>18</sup>

Various types of tetrazolium components are used to detect cell viability. It was divided into 2 categories, namely: 1) MTT, which is positively charged and readily penetrates viable eukaryotic cells, and 2) those such as 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethylphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-sulfophenyl)-2H tetrazolium, monosodium salt (WST-1) which are negatively charged and do not readily penetrate cells. MTT assay is a popular method used in various biomedical research. The MTT assay was developed in 1983 by Mosmann T et al. to detect cell viability using an MTT reagent.<sup>16,19</sup>

The MTT reagent, also known as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide, is a mono-tetrazolium salt comprising a positively charged quaternary tetrazole ring core with four nitrogen atoms. This core is encircled by three aromatic rings, including two phenyl groups and one thiazolyl ring. When MTT undergoes reduction, the central tetrazole ring is

disrupted, creating a violet-blue water-insoluble compound known as formazan.<sup>6</sup>

The resulting formazan was then dissolved, and its absorbance was measured using a spectrophotometer. Higher absorbance indicates higher cell viability. MTT assays can be used to evaluate the effects of various conditions and treatments on cells in-vitro, such as testing the effects of drugs or plant extracts on cells. This method is relatively fast, sensitive, and automatable for analyzing various biomedical and pharmaceutical research cell types. Besides being used to determine cell viability, the MTT assay is also used to study the toxicity of a substance. In the toxicity test, the compound to be tested is added to the cell culture, and then an MTT assay is performed. If the compound has a toxic effect on cells, cell viability will decrease and decrease the absorbance of the resulting formazan. This decrease in absorbance can be used as an indicator of a toxic effect on the tested cells. MTT assay can assist in the assessment of the level of toxicity of a substance and provide important information in the field of toxicological research and drug development.<sup>18</sup>

Besides being used to assess cell proliferation, cell viability, and toxicity of a substance, the MTT assay is also used to assess the activity of cell metabolic and mitochondrial activity.<sup>7</sup> Feng CQ et al. stated that the MTT assay proves to be a reliable method for identifying apoptotic cells, enabling the clear differentiation of apoptotic cells from both live and dead cells in-vitro.<sup>20</sup>

Our study identified the potential for hyperglycemia to reduce myoblast cell viability using the MTT assay method. Myoblast cell viability was identified to decrease due to administration of D-glucose at concentrations of 10 mM, 25 mM, and 50 mM. These results are supported by research conducted by Binjawhar DN et al., where there is a decrease in cell viability based on the results of the MTT assay on endothelial cells due to exposure to glucose. This indicates the induction of cell damage due to hyperglycemia.<sup>21</sup> Significant reduction in cell viability caused by hyperglycemia indicates a pro-oxidative stress environment created by the high glucose

concentration. Several studies revealed that several genes are highly expressed and related to pro-diabetic, pro-inflammatory, and pro-oxidative stress.<sup>22-25</sup>

Studies using animal models have been carried out to identify the detrimental effect caused by hyperglycemia and diabetes on skeletal muscle cells. Research has found a decrease in cell quality as indicated by changes in cell function, morphology, and metabolism. In addition, hyperglycemia is thought to interfere with the process of skeletal muscle regeneration, so the muscles lose their ability to maintain their functional capacity. Other studies have also strengthened the notion that uncontrolled diabetic conditions will alter the muscle progenitor cell population, which is identical to skeletal muscle disorders in diabetic patients.<sup>26-31</sup>

A previous study by Badu-Mensah A et al. showed a decrease in myoblast cell proliferation in a D-glucose concentration-dependent manner. In addition, hyperglycemia is also known to affect myoblast cell cycle transversal from S and G2/M phases.<sup>15</sup>

Hyperglycemia can induce ROS formation in mitochondria and is a major cause of hyperglycemic complications. Research conducted by Yu T et al. suggested that the hyperglycemic environment contributes to progressive mitochondrial fragmentation by activating mitochondrial fission by intracellular Ca<sup>2+</sup> and extracellular signal-regulated kinases 1/2. The initial experiment involved subjecting a liver cell line and a cardiac myoblast cell line of rats to elevated glucose levels. This led to increased reactive oxygen species (ROS) production in the mitochondria, as determined by fluorescent dyes such as fluorescein and dihydroethidium. The elevated ROS production resulting from hyperglycemia was accompanied by a significant alteration in the shape of the mitochondria, as they displayed fragmentation facilitated by the process of mitochondrial fission. In contrast, when the cells were exposed to L-glucose, a stereoisomer that cannot be transported or metabolized into pyruvate by the cells, there was no increase in ROS production or induction of mitochondrial fragmentation. This supports the idea that glucose metabolism is crucial for inducing

changes in mitochondrial morphology and generating ROS.<sup>32,33</sup>

A study revealed the potential mechanism of the fragmentation of mitochondria as a response to hyperglycemia conditions. Hyperglycemia triggers the activation of the mitochondrial permeability transition pore, releasing outer membrane permeabilization proteins and apoptotic factors like cytochrome-C and caspase. This process ultimately leads to cell death through apoptosis.<sup>34-36</sup> The research also demonstrated that inhibiting mitochondrial fission and promoting mitochondrial fusion can prevent the excessive formation of ROS (reactive oxygen species) under hyperglycemic conditions. These results imply that mitochondrial fission is crucial in regulating ROS production during cell death caused by hyperglycemia. Consequently, targeting the machinery responsible for mitochondrial fission could be a promising approach to mitigate ROS-related complications associated with hyperglycemia.<sup>37-39</sup>

## CONCLUSION

In conclusion, our study reveals that hyperglycemia leads to a notable decrease in myoblast cell viability, as evidenced by the MTT assay. The findings indicate that elevated glucose levels have a detrimental impact on myoblasts, potentially compromising muscle health. The MTT assay proved to be a valuable tool for accurately assessing myoblast cell viability in hyperglycemia. This study did not examine the apoptotic process of myoblast cells due to the decrease in cell viability from the MTT assay results, so this needs to be done in further research.

## CONFLICT OF INTEREST

The author reports no conflicts of interest in this work.

## ETHICAL CONSIDERATION

This research has been approved by the Ethical Committee of Research in Medical Health, Faculty of Medicine, Public Health, and Nursing of Universitas Gadjah Mada, Yogyakarta, Indonesia (Reference number: KE/FK/0600/EC).

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## AUTHORS CONTRIBUTION

DMR: concept, design, definition of intellectual content, literature search, experimental study, data acquisition, manuscript preparation, manuscript review, guarantor. JS: definition of intellectual content, literature search, experimental study, data acquisition, data analysis, statistical analysis, manuscript preparation. SS: literature search, experimental study, data acquisition, manuscript preparation, manuscript review

LDA: literature search, experimental study, data acquisition, manuscript preparation, manuscript review. NS: literature search, experimental study, data acquisition, manuscript preparation, manuscript review.

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