Effect of amlodipine on cytosolic calcium and apoptosis in hyperglycemia induced-human neuronal cell line culture

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Research note

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Abstract

Objective

Diabetic neuropathy is complication of diabetes which is believed to be caused by improper Ca^{2+} (calcium) signaling and activation of apoptotic process of neuron of dorsal root ganglia. Amlodipine, antagonist Ca^{2+}, has been already known to has neuroprotective effect in vitro or in vivo. This research investigates the effect of amlodipine in cytosolic calcium level and apoptosis in SH-SY5Y human neural cell line after exposure of chronic hyperglycemia. The study design was an experimental study using human neuronal cell line SH-SY5Y, exposed by chronic hyperglycemia for 6 days with concentration of glucose 25 μM (normoglycemia) and 50 μM (hyperglycemia), then was added amlodipine 2 μM for 30 minutes.

Results

In this study, hyperglycemia increased calcium concentration and caspase-3 compared with normoglycemia ($p = 0.004$ and $p = 0.001$ respectively). There was significant difference ($p = 0.015$) between calcium concentration in hyperglycemia induced cell line after given amlodipine 2 μM compared without amlodipine. There was significant difference ($p = 0.027$) between caspase 3 level in hyperglycemia induced cell line after given amlodipine 2 μM and without amlodipine. Administration of amlodipine significantly reduced cytosolic calcium and caspase-3 level in hyperglycemia induced SH-SY5Y human neural cell lines.

Introduction

Diabetic neuropathy is a complication of chronic diabetes, which can affect more than 60% patients. The pathogenesis of the disease is still not well understood. Classic mechanism that has been proposed is hyperglycemia will activate metabolic pathways such as polyol pathway, protein kinase-C pathway, advanced glycation end product and hexosamine pathway. All these pathways will lead to diabetic neuropathy complication through the production of reactive oxygen species (ROS) in the mitochondria. This condition will trigger long term accumulation of nerve injury that cause neuropathy [1].

Diabetic neuropathy can induce abnormal Ca^{2+} signalling which manifest to the increase of resting Ca^{2+} in intracellular, decrease activation of Ca^{2+} transport. Hyperglycaemia could slow nerve conduction, increase oxidative stress and activation of apoptotic pathways. This abnormality can be found in the experimental rat with diabetes [2]. The neuroprotective effect of the dyhidropiridine Ca^{2+} antagonist (amlodipine besylate and amlodipine camsylate) has been believed to reduce cell death due to oxidative stress [3]. In this study, amlodipine administration is expected to reduce cytosolic Ca^{2+} and apoptosis of hyperglycemia induced neuronal cells.

Methods

Cell culture

This experiment used Human neuroblastoma cell line SH-SY5Y from Cell Lines Servive (CLS) with catalogue number 300154. We used 24 wells to give exposure to the cell lines. We did cell thawing to melt the frozen cell until it was ready to be passage. Cell passage will produce cells in flask media, which was ready to be platted in 24 wells. In the flask media, cells were grown with media rich nutrition and antibiotic (L-glutamin, penstrep, Fetal Bovine Serum (FBS), and Dulbecco's Modified Eagle Medium (DMEM) high glucose) until it was confluent. FBS and DMEM were obtained from Thermofisher Life Technologies, catalogue number 1566368 and 11965092. The next step was centrifugation and media changing, then cells were plated in wells.

Hyperglycemia induction and amlodipine administration

Hyperglycemia induction was done by giving glucose 25 mM and 50 mM for 6 days. We observed culture growth and changed the medium every 2 days. After 6 days, we administer amlodipine to the cell culture (from Santa Cruz Biotechnology, catalogue number CAS 88150-42-9) with dose 0 and 2 μM, for 30 minutes. After giving amlodipine, we rinsed and fixated cell culture.

Staining and microscopic examination

To measure calcium level, cells were stained with fluo-3 (from Sigma-Aldrich, catalogue number 46393, CAS number 123632-39-3) in a dark room. On the other hand, to measure caspase-3 after fixation cells were stained with antibody caspase-3 (from Santa Cruz, catalogue number Sc-7272) for 2 days. After stained, the next process was cell observation with microscope confocal Olympus type FV1000 with software Olympus Fluoview Ver. 4.2a. Observation was done by measuring fluorescence cell. Cells which expressed calcium showed green color, meanwhile caspase-3 expression was seen in red color (rhodamin absorbent).

Statistical analysis

Results are expressed as mean. Independent T Test was used for comparisons of cytosolic calcium level and caspase-3 level between nomoglycemia and hyperglycemia cell before and after giving amlodipine. The Pearson Correlation test was used to know the relationship between calcium and caspase-3 level. P values < 0.05 were considered as significant value. Data analysis used SPSS 22.0.

Results

This experiment was carried out to see the effect of amlodipine on the calcium expression of hyperglycemia neuron cell groups. With amlodipine, the average calcium cytosol expression of hyperglycemia cells is higher than normoglycemia group. Different test results (independent T Test) produced a p value of 0.015, which showed a significant difference in the expression of cytosolic calcium in hyperglycemia SH-SY5Y cells treated with amlodipine and without amlodipine. Amlodipine

administration decreases the average calcium cytosol expression in hyperglycemias cells, can be seen in Fig. 1.

Subsequent experiments were conducted to see the effect of amlodipine on the expression of caspase-3 groups of hyperglycemia neuron cells. With the administration of amlodipine, the mean expression of caspase-3 in hyperglycemia cells is higher than in normoglycemia cells. Different test results (independent T Test) showed a value of p = 0.027 which showed a significant difference in caspase-3 expression in hyperglycemia SH-SY5Y cells treated with amlodipine and without amlodipine. Amlodipine administration decreases the mean expression of caspase-3 in hyperglycemias cells, can be seen in Fig. 2.

We also analysed correlation between calcium and caspase-3. Pearson correlation was used to measure it. It showed that there was significant correlation between calcium and caspase 3 level in hyperglycemia induced cells ($p < 0.001$, $r = 0.98$). In hyperglycemia induced cells after given amlodipine, we also analyse it by peasron correlation. It showed that there was significant correlation between Ca^{2+} and caspase-3 level ($p < 0.001$; $r = 0,985$ (strong correlation)). Figure 3 shows the average calcium and caspase-3 expression, seen in the hyperglycemia (glucose 50 mM) group without amlodipine, calcium and caspase-3 expression increased with 50 mM hyperglycemia exposure. Amlodipine 2 μM administration in the glucose 25 mM (normoglycemia) group and glucose 50 mM (hyperglycemia) group showed calcium and caspase-3 expression significantly decreased.

Discussion

Hyperglycemia effect in cytosolic calcium and caspase-3 expression of neuronal cell SH-SY5Y
In this study, we used human neuronal cell line SH-SY5Y which was induced by chronic hyperglycemia condition. Expression of Ca^{2+} in normoglycemia cell compared to hyperglycemia cell was significantly different ($p = 0.004$). Hyperglycemia induced cell had higher levels of Ca^{2+} than normoglycemia cell. This result similar to study by Verkhratsky, 2008, which revealed that chronic glucose exposure induced elevation of Ca^{2+} intracellular of neuron [4, 5]. Boldizsar et al., stated that hyperglicemia could increase cytosolic calcium level in human T cell [6].

Caspase-3 is terminal executor of the apoptotic pathway. In our study, hyperglycemia induced SH-SY5Y increased caspase level by 19.26 compared to normoglycemia cell (10.46). Statistical analysis showed a significant difference with p value = 0.000. This result similar to the study by Sharifi et al., which revealed that apoptosis induced by hyperglycemia (glucose 100 mM) in cell line PC12 correlated with activation of caspase-8, -9 and − 3 [7]. Vincent et al., stated that after hyperglycemia induction, the dorsal root ganglia neuron would show mitochondrial depolarization, release of cytochrome-c, activate caspase degradation deoxyribonucleic acid (DNA) protein and condensed chromatin. 30% of diabetic rat would increase caspase-3 level. [8]. Ortiz et al., reported that renal tubular cell induced by hyperglycemia could lead to apoptotic process through expression of Bcl-2-associated X (BAX) and downregulation of Bcl-2 and Bcl-Xl [9]. Federici et al., revealed that islet pancreatic cell, after induction of hyperglycemia, could lead to

apoptotic process through upregulation of BCL2 associated agonist of cell death (BAD), BH3 interacting domain death agonist (BID) protein, and downregulation of Bcl-Xl [10]. Nakagami et al., showed that hyperlgycemia induced endothelial cell would promote apoptosis through translocation of BAX to mitochondrial membrane and activation of caspase-9 and caspase-3 [11].

Effect of amlodipine on cytosolic calcium expression

Expression of Ca^{2+} in hyperglycemia cell and hyperglycemia cell plus amlodipine was significantly different with p value = 0.015. There was a decrease expression of Ca^{2+} in hyperglycemia cell after giving amlodipine. Amlodipine is third generation of dihydropiridine Ca^{2+} antagonist which is used as an antihypertensive agent. Amlodipine has various neuroprotective effects in vitro and in vivo model of cerebral ischemia. Amlodipine also has role to prevent neuronal damage caused by ischemia in rat [12]. Lee et al., reported that there was a neuroprotective effect of the dyhidropiridine Ca^{2+} antagonist (amlodipine besylate dan amlodipine camsylate) in reducing cell death due to oxidative stress [3].

A study by Warnock et al., reported that in ceroid lipofuscinosis neuronal 3 (CLN3) disease, there is neuronal degeneration that related to the elevated intracellular calcium showed by elevation of etoposide induce in terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Amlodipine significantly reduces intracellular Ca^{2+} in CLN3 protein inhibited cell and switch to normal level shown in the control [13].

The neuroprotective effect of calcium channel blocker (CCB) correlates with their ability to modulate intracellular calcium level, which is the one of signalling mechanism in apoptosis. Elevation of intracellular calcium level will activate endonuclease enzyme involved in DNA fragmentation. Another mechanism is calcium change conformation nuclear chromatin, so it is easy to be upregulated by specific endonuclease that cause DNA fragmentation. Elevation intracellular calcium correlates with the increase of free radical (stimulus of apoptosis). A free radical is the product of phospholipase A2, nitric oxide (NO) synthetase and xanthine oxidase activation. Inhibition of calcium channel will reduce cytosolic calcium level and glutamate release so that apoptotic process decrease [14].

Effect of amlodipine on caspase-3 expression

In this study, there was significant difference caspase-3 level in hyperglycemia cell compared with hyperglycemia plus amlodipine (p = 0.027). Amlodipine administration in hyperglycemia induced cell reduced caspase-3 level. Zhou et al., 2015, conducted studies in beta cell pancreas. Overload insulin secretion could induce cell to apoptotis process. Inhibition of Ca^{2+} influx could prevent endoplasmic reticulum (ER) from stress and prevent from cell apoptosis. Beta cell after exposing by free fatty acid and hyperglycemia, will progress to activate c-Jun N-terminal kinase (JNK) pathway which could lead to ER stress and cell apoptosis [15].

Another study by Park et al., 2019, who conduct research on the neuroprotective effect of amlodipine in neural stem cell induced by oxygen glucose deprivation (OGD). Amlodipine significantly increase expression of mitochondrial biogenesis –related protein and mitochondria anti apoptosis protein. Therefore, amlodipine could protect OGD injured NSC by maintaining function and structure of

mitochondria. Amlodipine protected OGD –injured NSC by inhibiting mitochondrial calcium influx, activate the PI3K pathway and maximized protein biogenesis [16].
Correlation of intracellular calcium and caspase-3 expression in SH-SY5Y cell induced by hyperglycemia
Our study showed significant correlation between intracellular Ca^{2+} and caspase-3 expression of SH-SY5Y cell induced by hyperglycemia ($r = 0.098$, $p < 0.001$). R value was positive, indicate that the higher level of intracellular Ca^{2+}, the higher level of caspase-3. This could be related to the pathophysiology that hyperglycemia induce apoptosis affected by many pathways. Study about homeostasis Ca^{2+} in apoptosis showed that it related to Bcl-2 protein. Bcl-2 has been detected in the outer membrane of mitochondria, ER, nucleus and cytoplasm. Principally, Ca^{2+} reduction in ER activates influx of Ca^{2+}, so that inducing long term Ca^{2+} elevation which lead to apoptosis mediated by Ca^{2+} [17].

Our study also showed a significant relation between intracellular Ca^{2+} and caspase-3 level in hyperglycemia induced cell after is given amlodipine ($r = 0.985$, $p = 0.000$). It has been reported antioxidant effect of dyhidropiridine calcium channel blockers. This related to the effect of mitochondrial protection in pathological conditions such as ischemia or hypoxia. Amlodipine inhibits L-type calcium channel so that can prevent cell from death [18].

Conclusion

Chronic hyperglycemia induction increases cytosolic calcium level and cell apoptosis. Amlodipine administration in human neuronal cell culture SH-SY5Y cell line induced by hyperglycemia reduces cytosolic calcium level and cell apoptosis. There was significant correlation between cytosolic calcium level and apoptosis in hyperglycemia cell and in hyperglycemia cell after giving amlodipine.

Limitations

- Further research needs to be done with more amlodipine dose variants much to get more consistent results.
- It is necessary to observe the neuron cell culture in the first days of treating hyperglycemia, thus proving the theory that the expression calcium and caspase-3 increased in acute hyperglycemia and decreased in chronic hyperglycemia.

Abbreviations

ROS: reactive oxygen species; FBS: fetal bovine serum; DMEM: Dulbecco's Modified Eagle Medium; BAD: BCL2 associated agonist of cell death; BID: BH3 interacting domain death agonist; BAX: Bcl-2-associated X; NO: nitric oxide; DNA: deoxyribonucleic acid; CCB: calcium channel blocker; CLN3: ceroid lipofuscinosis neuronal 3; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling; OGD: oxygen glucose deprivation; JNK: c-Jun N-terminal kinase; ER: endoplasmic reticulum

Declarations

Ethics approval and consent to participate

All experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the University of Brawijaya. The approval number for experiments is 685-KEP-UB.

Availability of data and materials

Data are all contained within the paper. The datasets from the analyses are available from the corresponding author on reasonable request.

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Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SNK: study concept and design, funding, data analysis and interpretation, manuscript writing, editing and submission; ADC: data analysis and interpretation, performance culture techniques, manuscript writing. MH and DR: data analysis and interpretation and editing. All authors read and approved the final manuscript.

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Figures

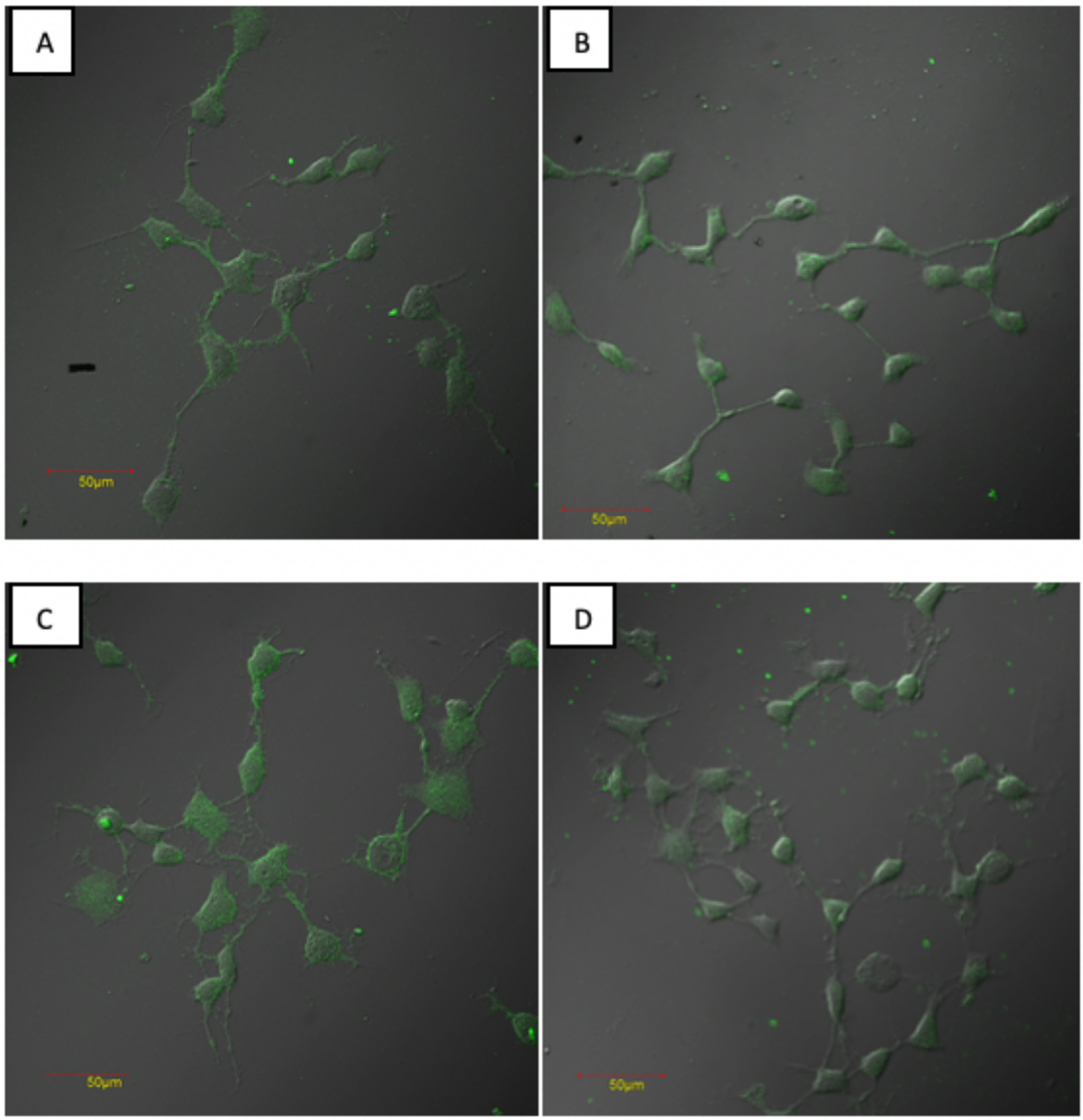

Figure 1

Calcium expression with immunohistochemical technique for each treatment group at 6th day. A. Normoglycemia (glucose 25 mM) without amlodipine B. Normoglycemia with amlodipine (glucose 25 mM with amlodipine 2 µM) C. Hyperglycemia (glucose 50 mM) without amlodipine D. Hyperglycemia with amlodipine (glucose 50 mM with amlodipine 2 µM). Calcium staining, green color, superimpose, magnification 400x.

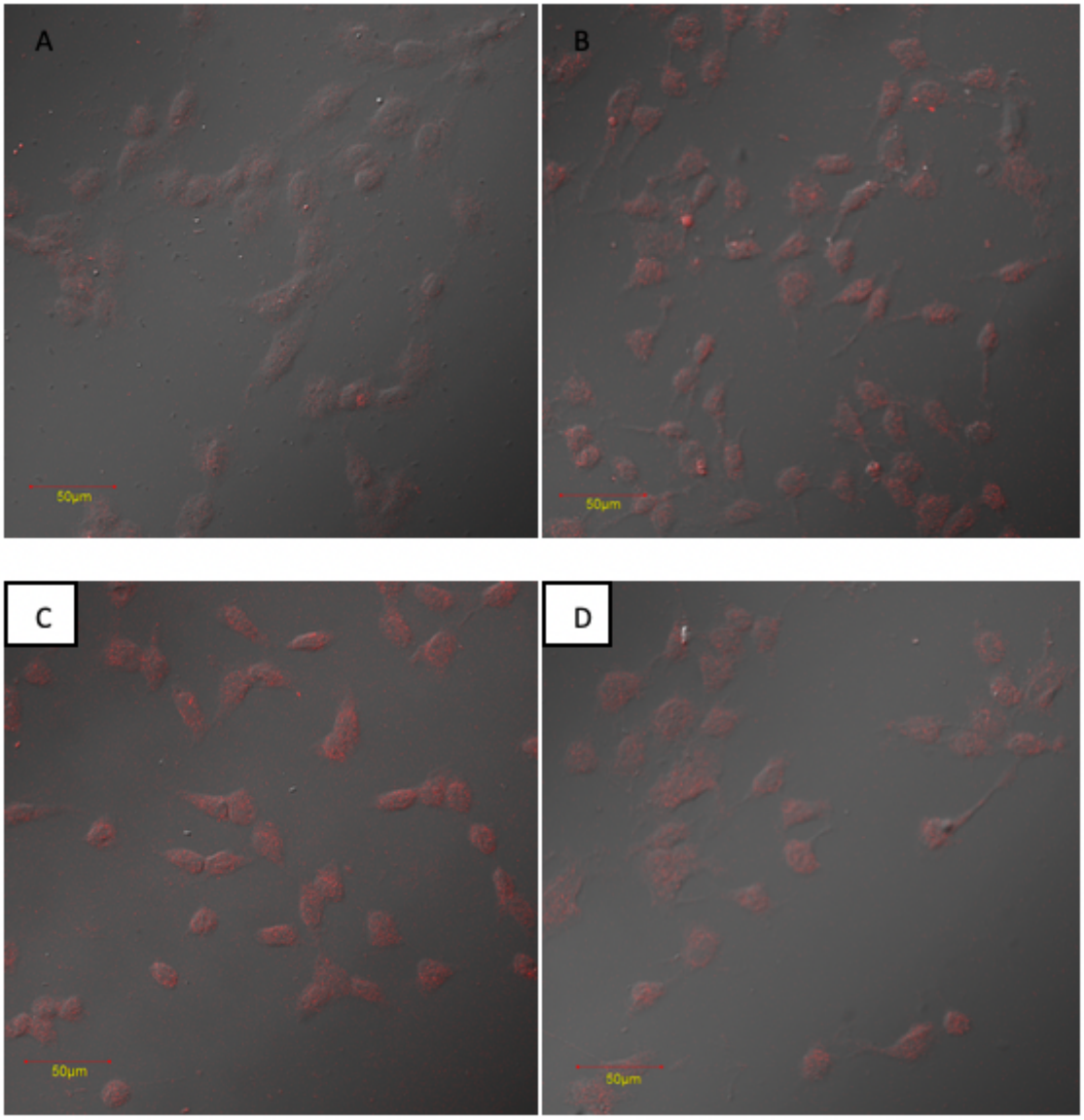

Figure 2

Caspase-3 expression with immunohistochemical technique for each treatment group at 6th day. A. Normoglycemia (glucose 25 mM) without amlodipine B. Normoglycemia with amlodipine (glucose 25 mM with amlodipine 2 µM) C. Hyperglycemia (glucose 50 mM) without amlodipine D. Hyperglycemia with amlodipine (glucose 50 mM with amlodipine 2 µM). Caspase-3 staining, red color, superimpose, magnification 400x.

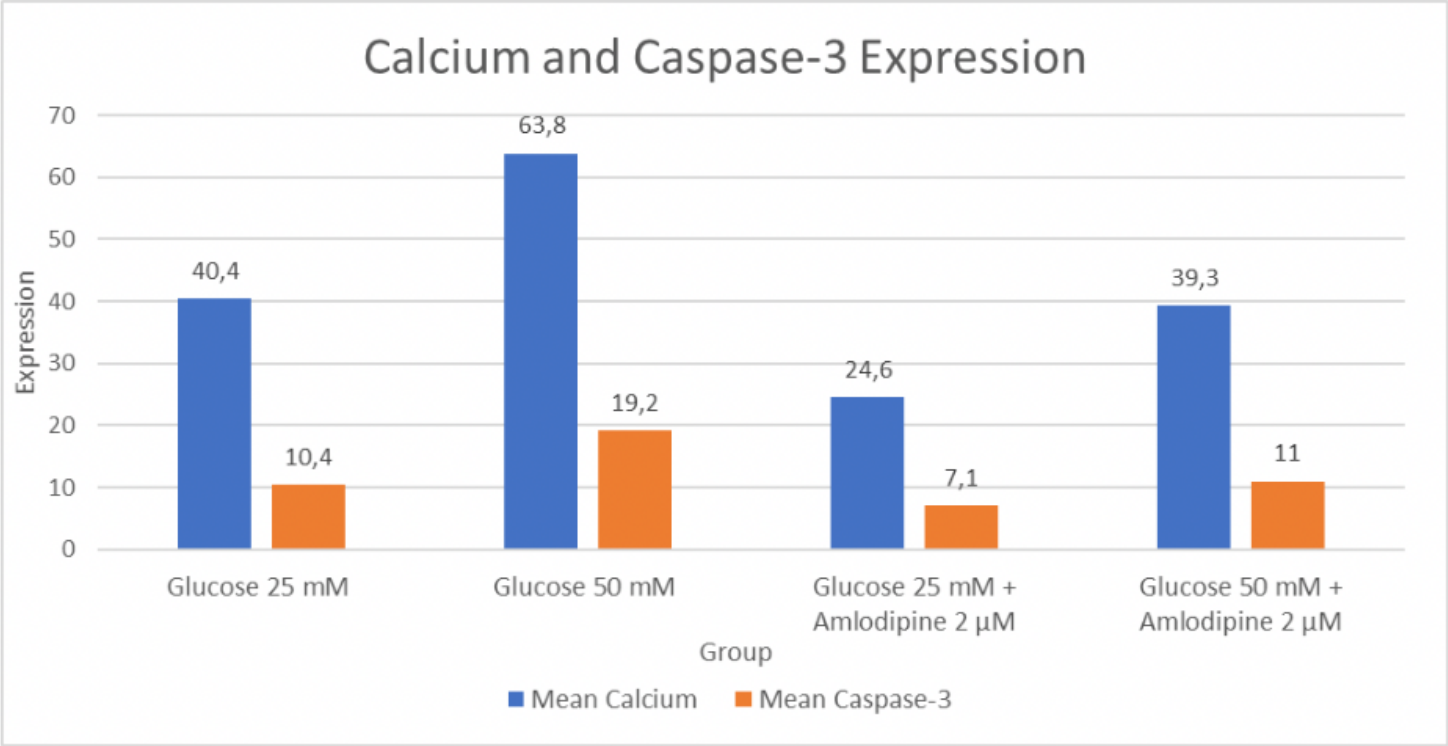

Figure 3

Mean of calcium and caspase-3 expression. Calcium and caspase-3 expression were highest at hyperglycemia (glucose 50 mM) group, while hyperglycemia (glucose 50 mM) with amlodipine 2 µM group has more decrease mean of calcium and caspase-3 expression.