



Differential expression of glycogen synthase kinase 3 α and 3 β isomers in brain cortex of mice following high doses of glucose

Mohd Alaraj^{*1,2}, Irena Kosinska³, Bahaa Deen Al-Trad⁴, Ammar Almaaytah^{5,1}, Tarek D. Hussein⁶, Ashfaque Hossain⁷, Mohamed Jamal Saadh¹

¹Department of Pharmacy, Faculty of Pharmacy, Middle East University, Amman, Jordan

²Department of Pharmacology, College of Medicine, University of Hail, Kingdom of Saudi Arabia

³Department of Social Medicine and Public Health, Medical University of Warsaw, Poland

⁴Department of Biological Sciences, Yarmouk University, Irbid, Jordan

⁵Department of Pharmaceutical Technology, Faculty of Pharmacy, Jordan University of Science and Technology, Irbid, Jordan

⁶Department of Zoology, Faculty of Science, Cairo University, Egypt

⁷Department of Medical Microbiology, Ras Al Khaimah Medical and Health Sciences University, Ras Al Khaimah, UAE

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ABSTRACT

Glycogen synthase kinase 3 (GSK3) encodes a serine/threonine protein kinase. We investigated the effects of Subcutaneous (SC) glucose administration on the expression of glycogen synthase kinase 3 (GSK-3) isomers (α and β) genes in the cerebral cortex of mice with the aim of determining the possible mechanism(s) involved in mitochondrial dysfunction induced by hyperglycemia. Adult male BALB/c mice were treated with 12 gm/kg glucose solution SC once daily for 3 days. Ultrastructure study, histopathological analysis, and Real-time PCR investigations were carried out on the cerebral cortex from glucose treated mice and from vehicle-treated control animals. We observed significant ultrastructural damage of mitochondria in the cerebral cortex of mice received high doses of glucose. Histopathological alterations in the cortex of these animals were also detected. A significant increased of GSK-3 α gene expression and decreased expressions of GSK-3 β gene in high glucose treated animals were noticed. The hyperglycemia-induced ultrastructural changes may occur via modulation of gene expression of GSK-3 isomers, and we hypothesize this as an early etiopathological factor in hyperglycemia-related neurodegenerative diseases (NDD).



*Corresponding Author

Name: Mohd Alaraj

Phone: +962798279131

Email: malaraj@meu.edu.jo

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INTRODUCTION

Mitochondrial dysfunction, which may come about by numerous factors including Ca⁺² overload, oxidative stress, elevated phosphate concentration, and adenine nucleotide depletion, is a key mechanism of cell death (Pérez *et al.*, 2018). Indeed, the association between dysfunction of mitochondria and cell loss has been well established in various diseases, including NDD, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and Amyotrophic lateral sclerosis (Hawkins and Duchon, 2019; Chen *et al.*, 2019). In parallel, several recent

findings have indicated the involvement of diabetes mellitus in the induction of NDD, i.e., Alzheimer's and Parkinson's diseases (Intihar *et al.*, 2019; Morsi *et al.*, 2018). However, most of the previous studies concentrated on the role of insulin deficiency or insulin resistance in exhibiting these abnormalities (Macklin *et al.*, 2017; Monte *et al.*, 2017; Nameni *et al.*, 2017), but only a few investigated the implication of hyperglycaemia in diabetes mediated neurodegeneration (Kumar *et al.*, 2017). Current studies propose that hyperglycaemia, regardless of its origin, is a leading factor in inducing diabetic linked brain neurodegeneration (Iadecola, 2015; Wongchai *et al.*, 2015). By this reckoning, it was suggested that both diabetic and non-diabetic linked hyperglycaemia contributes to neuronal cell death leading to neurodegeneration (Quincozes-Santos *et al.*, 2017). Studies have linked diabetes mellitus and insulin resistance with impaired brain and peripheral neuronal mitochondria. The underlying mechanism being diminished activity of complexes III, IV and V of the respiratory chain and adenosine triphosphate due to increased oxidative and nitrosative stress. (Maciejczyk *et al.*, 2019; Peng *et al.*, 2016; Alaraj *et al.*, 2004). Calorie restriction has been shown to have a positive effect on respiratory capability of mitochondria of brain. In addition, calorie restriction also demonstrated to prevent neuronal loss. (Alaraj *et al.*, 2018; Apple *et al.*, 2019).

Accumulating evidences argue that GSK-3, particularly β isomer, is implicated in cell loss through decreasing the threshold for the opening of the mitochondrial mega channel (Yang *et al.*, 2017). At the same time, many studies demonstrated the involvement of GSK-3 β in several NDD (Jaworski *et al.*, 2019; Thomas *et al.*, 2013; Matsunaga *et al.*, 2019; Shahab *et al.*, 2014; Sulaiman *et al.*, 2019).

Different GSK-3 inhibitors have been revealed to increase neurogenesis of human neural progenitor cells, and they demonstrate encouraging results for the treatment of these disparaging diseases (Jaworski *et al.*, 2019; Mathuram *et al.*, 2018). However, our present knowledge about the molecular mechanism that may link between hyperglycaemia and mitochondrial dysfunction is still lacking clear insights and requires more studies at the molecular level. Moreover, the potential role of hyperglycaemia in modulating the expression of GSK-3 isomers in the brain cortex has not yet been estimated. At the same time, the etiopathology of NDD is poorly understood. In light of the above, our major aim in the present study was to explore the potential effects of hyperglycaemia on gene expression of GSK-3 isomers. We used a murine model to carryout ultrastructural and histopatho-

logical analysis to sought answers to our question. Moreover, we used gene expression analysis of GSK-3 isomer to address possible molecular basis of observed changes.

MATERIALS AND METHODS

Study approval

The research project (No. EC0023) received approval from the Ethical Review Committee (ERC) of the College of Medicine, University of Hail, Hail, Kingdom of Saudi Arabia.

Animals

The mice were kept in separate cages assigned to different groups. The room was maintained at steady humidity and temperature and light-dark cycle. Prior approval of the experimental procedures was obtained from the animal care and use committee of the University of Hail. Experimental and control groups consisted of 6 mice in each. Mice were randomly assigned to the different groups.

Administration of glucose

Glucose solution in the dose of 12g/kg body weight was daily administrated SC to the experimental animals for three days. Control mice were given the same volume of normal saline for three days. After 1hour of the last dose, all animals were decapitated and used for experiments

Electron Microscopy

For electron microscopic examinations, the animals were anaesthetised intraperitoneally with 0.2-0.4ml of 20% urethane. In situ fixation of the brain was accomplished. Transcardiac perfusion was carried out with 0.1M cacodylate buffer (10 sec), followed by 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (2ml/ min). After removal of the brains, blocks of the cerebral cortex were maintained in the same fixative solution at 4°C for 2h and post-fixed in 2% osmium tetroxide in 0.1M cacodylate buffer for 2h at 4°C. The blocks of tissues were rinsed overnight in 0.1 M cacodylate buffer. The tissue blocks were dehydrated in increasing concentrations of propylene oxide and ethanol. After dehydration was complete, the tissue blocks were embedded in Epon. Ultrathome apparatus was used to prepare ultrathin sections, which were then post-stained with uranyl acetate and lead citrate. The prepared sections were examined using electron microscope (JOEL 1200EX).

Histology and Light microscopy

Ten percent neutralized formalin was used to fix the brains. The tissue samples were immersed in

a series of ethanol solutions followed by xylene (to remove the residual fat from the samples). Then the tissue samples were embedded in paraffin blocks. Sections (6 μ m thick) were cut and stained with hematoxylin-eosin for light microscopy (1X-71, Olympus, Tokyo, Japan).

RNA preparation and real-time RT-PCR

Total RNA was extracted from RNA later-preserved brain cortex tissues using an RNeasy mini tissue kit (Qiagen, USA) according to the manufacturer's protocols. The RNA pellets were dissolved in RNase-free water. The RNA samples were then quantitated by determining absorbance at 260 and 280 nm. The quality of the RNA samples was determined using 260/280 nm absorbance ratio. One-step quantitative real-time RT-PCR was carried out using the KAPA PROBE FAST One-Step qRT-PCR Master Mix (Kapa Biosystems, USA) using LineGene 9600 Real-Time PCR system (Bioer Technology Co, Bingjiang, China). PrimeTime[®] qPCR probe-based gene expression assays were purchased from Integrative DNA Technologies (Coralville, Iowa) for mRNAs of β -2-microglobulin (GenBank accession no. NM_009735; IDT Assay ID: Mm.PT.58.10497647), GSK-3 α (GenBank accession no. NM_001031667.1; IDT Assay ID: Mm.PT.58.6048580) and GSK-3 β (GenBank accession no. NM_019827; IDT Assay ID: Mm.PT.58.41280327). Relative mRNA concentrations were normalized to the housekeeping gene β -2-microglobulin.

Statistical analysis

SPSS version 16.0 for windows (SPSS Inc. Chicago, IL, USA) was used for statistical analysis. All numbered data were expressed as mean \pm SD. Independent samples t-test was used to identify differences between groups; a value less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Electron microscopic observations

Control mice which received normal saline showing normal mitochondria both outside and inside of axons (Figure 1-A).

Accumulation of glycogen granules in structurally altered mitochondria in brain of mouse injected high dose of glucose (12 g / kg body weight / day for 3 consecutive days).

One of the significant finding of this study came from the electron microscopic observations. Administration of high dose of glucose (12 g / Kg body weight / day for 3 consecutive days) resulted in visible, significant damage to the mitochondria of astrocytes

and synapses of the brain cortex. Fifty mitochondria were randomly selected among these mitochondria twenty six showed ultrastructure changes. Ultrastructural changes included condensation of mitochondrial matrix, lack of mitochondrial cristae and fragmentation of mitochondrial membrane. However, the brains of the control animals showed no such changes (Figure 1-B).

Histopathology

High dose of glucose induced histopathology, shrinkage and degradation of nerve cells showing little perineural vacuolation (Figure 2).

Expression of glycogen synthase kinase 3

Real-time polymerase chain reaction (rPCR) was performed in the cortex of the mouse, which was used for electron microscope experiments, i.e., animals which were injected with glucose in the dose of 12gm/kg body weight/day for three days and control mouse. We determined the effect of elevated intracellular glucose on the expression of glycogen synthase kinase-3 isoforms; α and β . As presented in Figure 3, the mRNA expression of GSK 3 α was significantly ($p < 0.05$) increased, but the expression of the β isoform was significantly decreased ($p < 0.05$).

Our present study, utilizing electron microscopy, has confirmed our previous observation regarding the ultrastructural changes in the mitochondria of the brain cortex of glucose treated mice (12gm/kg body weight/ day for 3 days). Our results are in line with several other studies which demonstrated that hyperglycemia causes mitochondrial dysfunction in various tissues, including brain mitochondria (Alaraj *et al.*, 2004; Zhiliuk *et al.*, 2015). Several studies showed that mitochondrial dysfunction occur early in the course of neuro degenerative diseases (NDD) and plays an important role in their pathogenesis. (Wang *et al.*, 2019; Hawkins and Duchon, 2019; Chen *et al.*, 2019; Intihar *et al.*, 2019; Lee *et al.*, 2017). Interestingly, in histopathological studies we observed significant morphological alteration in the brain cortex of mouse treated with high doses of glucose. To our knowledge, this work is the first research to evaluates the histologically behaviour of the brain cortex of healthy animals that administered with glucose at high doses. However, our histopathological findings were significantly less pronounced in comparison to STZ-induced diabetic rats obtained by other investigators (Huang *et al.*, 2012). This may be attributed to persistent hyperglycaemia, which accompanies STZ- induced diabetes mellitus, comparing with intermittent hyperglycaemia induced by administering high doses of glucose.

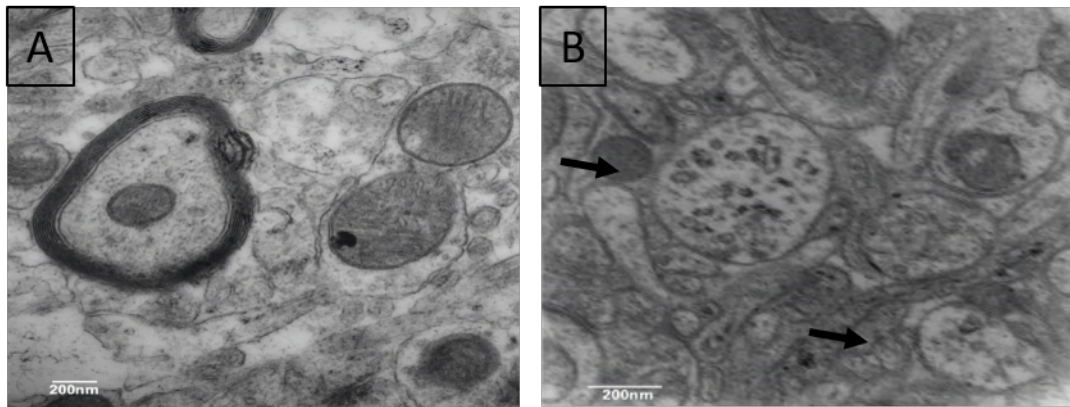


Figure 1: The brain cortex mice was under electron micrographs. A: After mouse had been given an injection contains normal saline, B: After mouse had been given an injection contains a high dose of glucose

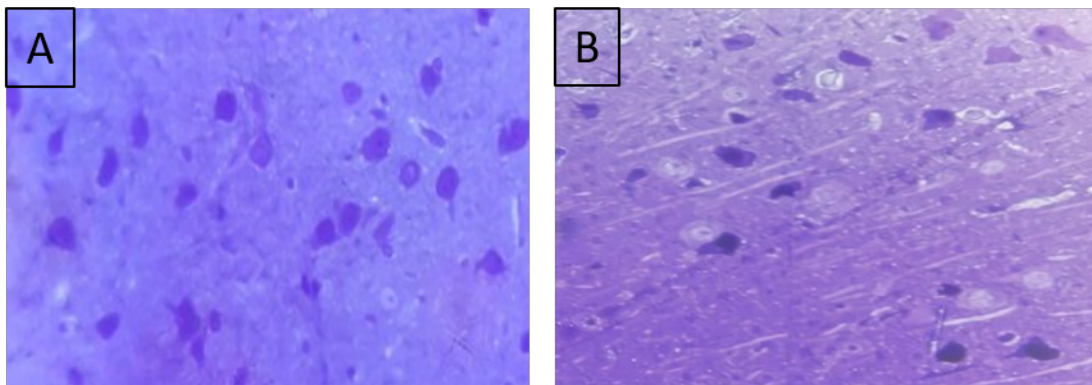


Figure 2: Brain cortex with neurodegeneration in mice due to hyperglycemia. A: Normal construction of brain cortex arrangement in the control mouse. B: Small sizes of some neurons, perineural vacuolation, in the cortex of brain mouse

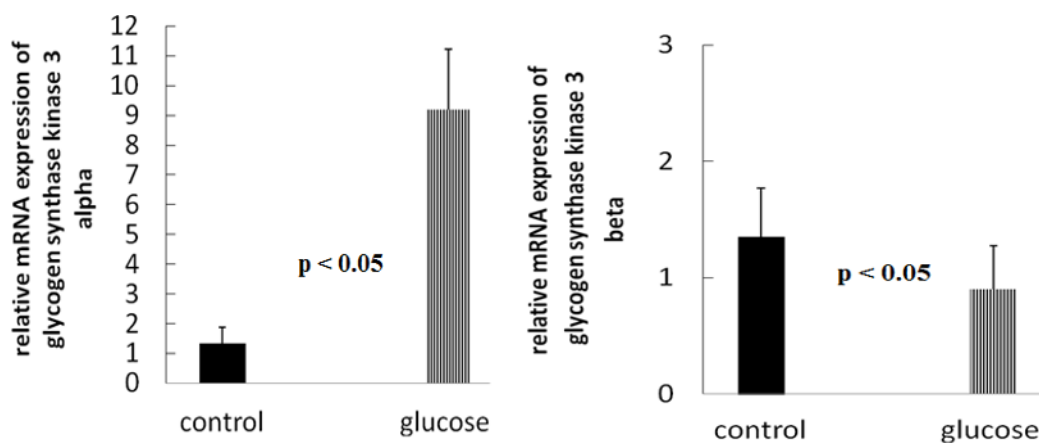


Figure 3: Real-time PCR detected that glucose treatment in the dose of 12g/kg body weight/day for three days, the expression of mRNA of GSK-3 α has $p < 0.05$, but the expression of mRNA of GSK-3 β has $p < 0.05$ in the brain cortex of treated

To explore the possible involvement of GSK-3 in hyperglycaemia-induced dysfunction of mitochondria, we utilized the real-time PCR technique to evaluate the impact of hyperglycaemia on the expression of both GSK-3 isomers, namely, GSK-3 α and GSK-3 β . It was found that inhibition of GSK-3/GSK-3 β has a significant positive impact on cerebral mitochondrial biogenesis (Theeuwes *et al.*, 2018).

Based on this study and that conducted by Tanaka, who demonstrated that GSK-3 and mainly the GSK-3 β isoform plays a key role in opening the mitochondrial mega-channels (Tanaka *et al.*, 2018), increased gene expression of this enzyme is expected to result in the induction of mitochondrial dysfunction. In the present study, however, we surprisingly observed that glucose given in high doses significantly decreased the expression of mRNA of GSK-3 β isoform but increased the expression of GSK-3 α , possibly demonstrating the implication of the later in hyperglycemia-induced mitochondrial impairment. Our results regarding the β isoform, are in agreement with that obtained by Thomas *et al.* (2013) who reported a reduction in gene expression of GSK-3 β in the hippocampus of STZ-induced diabetes mouse (Thomas *et al.*, 2013). However, the search of the literature revealed no data on the effects of high doses of glucose on GSK-3 in the mammalian brain cortex. On the other hand, increased expression of mRNA of GSK-3 α , which we observed in this study, is a novel finding, as other studies exclusively attribute neurodegeneration to GSK-3 β isomer (Jaworski *et al.*, 2019; Mathuram *et al.*, 2018). Off note, most investigations focused on GSK-3 β while ignoring α isoform, and only a few studies differentiate between these two proteins (Jaworski *et al.*, 2019; Mathuram *et al.*, 2018). Furthermore, available inhibitors, including lithium, inhibits both isoforms (Matsunaga *et al.*, 2019). Indeed, according to other studies, GSK-3 α isomer rather than β is involved in cognitive disorders, including Alzheimer's disease (Shahab *et al.*, 2014). Hence, it is possible to assume that GSK-3 α is involved in permeability of mitochondrial mega channel of the brain cortex rather than GSK-3 β isoform, which is responsible for this effect in mitochondria of cardiomyoblasts (Sulaiman *et al.*, 2019).

CONCLUSIONS

Taken together, the results obtained in this study indicate that hyperglycemia induced mitochondrial impairment might occur through elevating the gene expression of GSK-3 α , which in turn, can lead to cytotoxicity of brain cells and this may be as a

general molecular pathway through which hyperglycemia may induce NDD and thus serve as a new therapeutic target for these diseases. However, further investigations are needed to explore the role of reduced gene expression of GSK-3 β induced by hyperglycemia in the brain cortex.

To the best of our knowledge, this is the first study determining the modifying effect of hyperglycemia on the gene expression of GSK-3 isomers of brain cortex in rodent model.

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Ethical clearance

The study protocol conformed to the ethical guidelines of the 1975 Helsinki Declaration, and the approval was obtained from the ethical committee of Middle East University-Amman-Jordan.

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