Original Article

Insulin Can Improve the Normal Function of the Brain by Preventing the Loss of the Neurons

Mohammad Amin Tofighi Zavareh[1,2*], Mohammad Hossain Kargar Godaneh[1,3*], Parnian Eslahi[2], Abbas Aliaghaei[2], Mohammad Ali Asghari[1,3], Zahra Azimzadeh[1,3], Ali Asghar Payvandi[1], Ali Younesi[2], Mohammad Mahdi Gheibi[2], Fatemeh Alipour[2], Mohammad Amin Sabbagh Alvani[2], Mohammad Golshan Tafti[4], Mohammad-Amin Abdollahifar[1,2], Hojjat Allah Abbaszadeh[3,1**]**

Abstract

Background: Insulin promotes the expression of genes related to brain function, thus preventing the neurodegeneration process. The present study was designed to find the neuroprotective effect of insulin by reducing neuron loss in the brain.

Materials and Methods: In this study, 20 adult male NMRI mice were divided into two groups: control and insulin. The control group was intact, and the insulin group received 100 µL of insulin at a 72-hour interval by intraperitoneal (I.P.) injection for 30 days. At the end of the study, the brain was removed. The volume of the brain and the total number of neurons and glia were estimated by stereological techniques, and also the gene expression of NSR, PI3K, AKT, IGF-1, and FOXO-1 was measured using real-time PCR.

Results: The results showed that the total number of neurons decreased in the control group compared to the experimental group. Furthermore, the expression of NSR, PI3K, AKT, IGF-1, and FOXO-1 genes was lower in the control group than in the insulin group.

Conclusion: The results showed that treating mice with insulin prevented reducing the number of neurons and gene expression related to normal brain function. So, insulin could have neuroprotective effects against neuron loss. Insulin may be beneficial as a new approach to avoiding neuron loss in regenerative medicine.

Keywords: Insulin, Brain, Neurons Loss, Aging, PI3K-Akt Pathway

1. Hearing Disorders Research Center, Loghman Hakim Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran
2. Department of Biology and Anatomical Sciences, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
3. Laser Applications in Medical Sciences Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
4. Department of Pediatrics, Islamic Azad University of Medical Sciences, Yazd, Iran
***Co-First Authors**
****Co-corresponding Authors:** Mohammad-Amin Abdollahifar, Hearing Disorders Research Center, Loghman Hakim Hospital and Department of Biology and Anatomical Sciences, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran. P.O. Box: 19395-4719. Tel: (+98) 21 23872555; Fax: (+98) 21 22439976;
Email: m_amin58@yahoo.com
AND
Hojjat-Allah Abbaszadeh**,** Laser Application in Medical Sciences Research Center and Department of Biology and Anatomical Sciences, school of medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran. P.O. Box: 19395-4719. Tel: (+98) 21 23872555; Fax: (+98) 21 22439976.
Email: Dr.Abbaszadeh@sbmu.ac.ir

Please cite this article as: Tofighi Zavareh MA, Kargar Godaneh MH, Eslahi P, Aliaghaei A, Asghari MA, Azimzadeh Z, et al. Insulin Can Improve the Normal Function of the Brain by Preventing the Loss of the Neurons. J Cell Mol Anesth. 2022;7(4):197-202. DOI: https://doi.org/10.22037/jcma.v7i4.38324

Introduction

Over the years, people have been curious to find various novel methods for controlling the aging process and its consequences. Many studies have shown the effects of aging on brain function, metabolism, and structure, such as cognitive impairment, changes in the activities of neurotransmitters, and a decrease in brain size (1). However, the mechanism and pathways involved in the brain aging process are still unknown. There is a hypothesis that insulin may play a significant role in the brain aging process and may have a neurotrophic

function (2). It was considered that insulin could not pass the blood-brain barrier (BBB), so the brain was first demonstrated as an insulin-independent organ (1, 2). However, there was more insulin in the brain than in other organs (1-3). Insulin in the peripheral blood can cross the BBB by the saturated insulin receptor and free diffusion through porous capillaries in circumventricular regions (4).

Furthermore, it has been suggested that insulin can be produced within the brain (2). Due to the critical complexities in different parts of the central nervous system, many studies have been performed on chemical and medicinal compounds. Previous studies have shown that insulin may have a critical role in neural survival, neural plasticity, cognitive functions, and memory (5, 6). Recently, studies showed insulin signaling pathways are potential modulators in age-related neurodegenerative disease (4)**.**

Chronic oxidative stress can be an important cause of emerging brain aging and neurologic disorders (7). *Oxidative stress* is defined as exposure of a neural cell to reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the inadequacy of enzymatic (e.g., catalase, superoxide dismutase, glutathione peroxidase) and non-enzymatic (e.g., reduced glutathione (GSH), uric acid) anti-oxidative systems (8). It causes oxidative damage to different cell parts such as cell membranes or mitochondria, proteins, and even nucleic acid (9). Glutathione (GSH) is an essential brain antioxidant. It can also promote the regeneration of other free radical scavengers and regulate gene expression and neural apoptosis (10-12). Insulin through PI-3K/Akt and/or ERK signaling pathways stimulate the uric acid and glutathione redox cycle, thus limiting ROS activity (11). For many years, studies have been designed to determine the exact role of insulin on the brain aging process, but the main mechanism remains unknown. The present study was designed to find the neuroprotective effect of insulin by reducing neuron loss in the brain.

Methods

Ethical considerations: The protocol of the present study was reviewed and confirmed by the Ethics Research Committee, Shahid Beheshti University of Medical Sciences, Tehran, Iran (IR.SBMU.RETECH.REC.1399.1085).

Animals: In this experimental study, 20 adult male NMRI mice weighing 25–30g were obtained Pasteur Institute of Iran. The animals were set aside in animal households beneath standard conditions and provided with food, water, and ad libitum. The animals were then distributed into two groups: Group I (Control group): The animals of this group were intact. Group II (Insulin): The animals of this group received insulin at the dose of 100 µL at a 72-hour interval by intraperitoneal (I.P.) injection for 30 days. At the end of the study, the right hemisphere was extracted for stereology analysis, and the left hemisphere was used for molecular and cellular analyses.

Tissue preparation: At the end of week 33, all the animals were sacrificed by exposure to CO2 and then decapitated. Brain samples were removed and kept in Bouin solution for 48 hours. Paraffin-embedded samples were prepared to make 5 and 25 µm thick serial sections for volume estimation and neuronal counts, respectively. We selected ten sections of each sample using the Systematic Uniform Random Sampling (SURS) technique, and then they were stained with H&E stain.

Total Volume of the brain: We used the Cavalieri principle to estimate the brain's total volume with the following formula (13-15).

$$V = \sum P \times \frac{a}{p} \times t$$

Where ΣP is the total points hitting the brain sections, a/p is the area associated with each point, and t is the distance between the sampled sections.

Neuron and Glial cells count: To count the number of neurons and glial cells, we use the Optical dissector technique and the following formula for estimating the Numerical density (Nv).

$$N_v = \frac{\sum Q}{\sum P \times h \times \frac{a}{f}} \times \frac{t}{B.A.}$$

Wherever (ΣQ) is the number of cells and (ΣP) is the number of including frame grid in all fields; (a/f) is the area of frame; (h) is the dissector height; (B.A.) is the thickness of microtome section and (t) is the real section thickness (13-15).

Table 1: The sociodemographic characteristics and outcomes for gender.

Genes	Primer sequences
β-actin	F: TCAGAGCAAGAGAGGCATCC R: GGTCATCTTCTCACGGTTGG
INSR	F: GAGAGTGGTGGAGTTGAGTTGG R: TGTGGAGGATGGAGGAGGAG
PI3K	F: TAGCTGCATTGGAGCTCCTT R: TACGAACTGTGGGAGCAGAT
ATK	F: TCTGACGGGTAGAGTGTGCGT R: CTACTTCCTCCTCAAGAATGA
IGF-1	F: GGAAGCTATGGAGTGGGAAAAG R: CCGAGAGGTGGAGTGATTTGA
FOXO-1	F: AACTGAGGAGCAGTCCAAAGATG R: AACTGAGGAGCAGTCCAAAGATG

Analysis of NSR, PI3K, AKT, IGF-1, and FOXO-1, expression using real-time PCR: In subsequent extraction of total RNA samples, we preserved them with DNase I (Roche, Basel, Switzerland) to eliminate contagion made by genomic DNA. We used a profitable kit (Fermentas, Lithuania) to synthesize cDNA at 42˚C for 60 min in agreement with constructer orders. We used real-time PCR (TaqMan) based on the QuantiTect SYBR Green RT-PCR kit (Takara Bio Inc, Japan) to quantify the comparative expression of genes. Thoroughly pairs of reverse and forward primer were planned by Primer 3 Plus software using an exon-exon connection technique to depart cDNA from genomic DNA (Table 1) (16-18).

Statistical analysis: The measurable information was extracted from five independent samples and accessible as mean ± S.D. Statistical analyses were done by the SPSS software, version 20. Statistical significance was determined using the t-test and a two-way repeated-measures ANOVA. Statistical significance was set at $P<0.05$ (6).

This study was under the support of Shahid Beheshti University's Medical Research Ethics Committee (IR.SBMU.RETECH.REC.1399.1085).

Results

The volume of the brain: The study results revealed an increase in the volume of the brain in the insulin in comparison to the control group; however, the total volume of the brain showed no significant difference in the mice treated with insulin (Figure 1).

The number of neurons and glial cells: The results showed that the total number of glial cells increased in the brain. Still, these changes were not significantly different in the insulin group compared to the control group (Figures 1A, C, and D). The results showed that the total number of neurons in the brain was significantly increased in the insulin group in comparison to the control group ($P<0.01$) (Figure 1B, C, and D).

Real-time PCR analysis: The relative levels of mRNA expression NSR, PI3K, AKT, IGF-1, and FOXO-1 are normalized and quantified in various groups. As depicted below in Figure 2, the levels of NSR expression remarkably decreased in the control group when compared with the insulin groups ($P<0.01$) (Figure 2). Based on the results, a significant reduction was observed in levels of PI3K expression in the control group compared with the insulin group ($P < 0.001$) (Figure 2).

The expression of genes associated with apoptosis showed that insulin could have important effects in preventing the destruction of brain cells. In this study, the expression of AKT in the control group compared to the insulin group was significantly reduced ($P<0.001$) (Figure 2).

The expression of the IGF- gene in the control group compared to the insulin group significantly decreased ($P<0.001$) (Figure 2). Furthermore, the expression of the FOXO-1 gene in the control group significantly reduced when compared to the insulin group ($P<0.001$) (Figure 2).

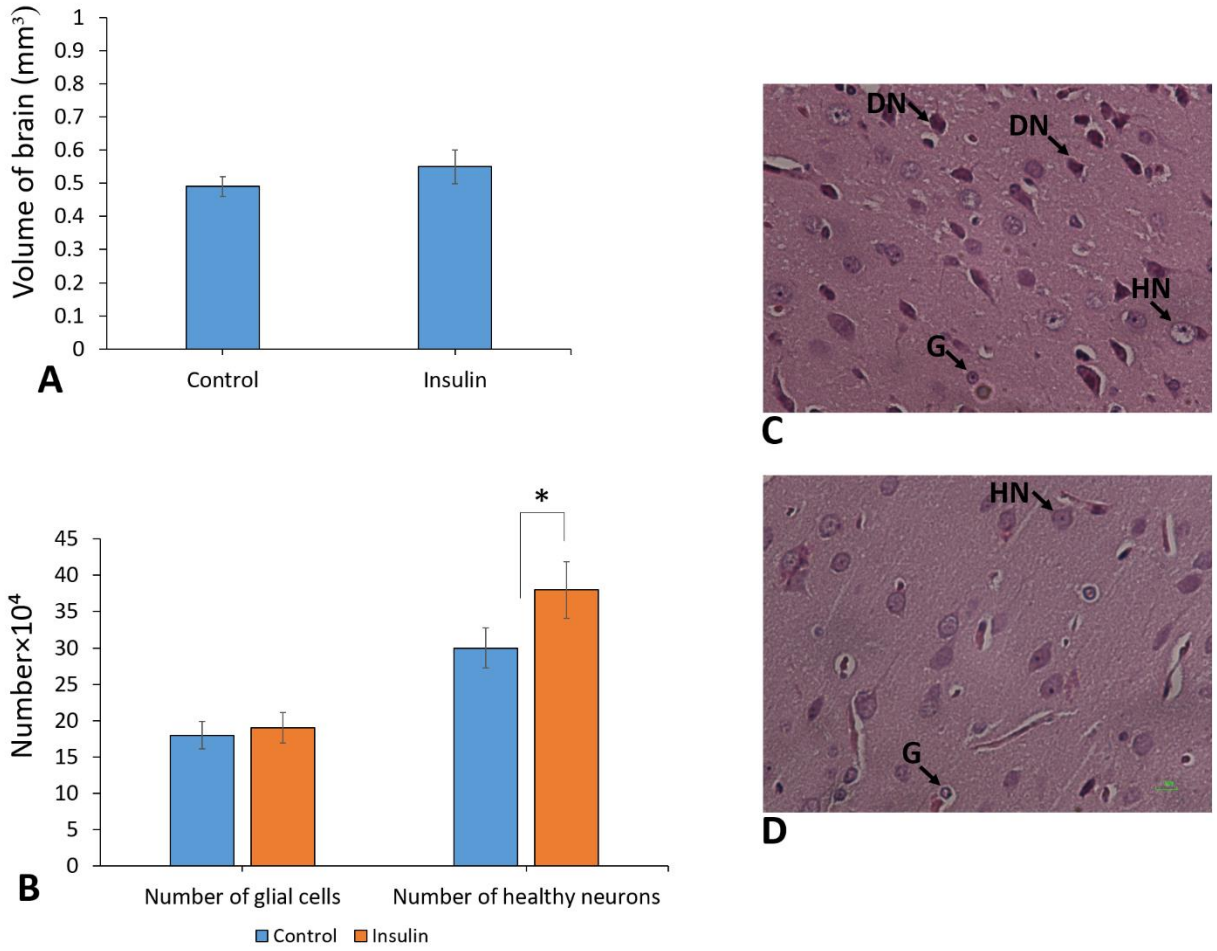

Figure 1. Cortical neuron and glial cell numbers in the brain cortex. (A) The total volume of the brain in the control, and insulin groups. A significant difference was not observed between the control and insulin groups. (B) The total number of neurons and glial cells in the control, and insulin groups. The significant difference between the control with the insulin groups is indicated. (*P<0.05). (C and D) Micrograph of the brain stained with hematoxylin and eosin (H&E); (C) control group, (D) insulin group; Dead neurons (DN); Healthy neurons (HN); Glial cells (G).

Discussion

Many studies have focused on finding a promising therapy against the brain aging process and age-related neurodegenerative diseases for many years. After the first detection of insulin and insulin receptors in the brain and numerous neurodegenerative diseases, it is suspected that insulin can be used as a treatment to postpone age-related disorders (13). Despite advancement in recognizing insulin's role in the brain, mechanisms of its engagement remain unknown (14), insulin receptors through the PI-3K/Akt and ERK signaling pathway have been discussed in previous studies as a probable mechanism of insulin activity in the cell apoptosis process (15). The present study tried to understand the neuroprotective effect of insulin by measuring the expression of genes that take part in the neuronal cell apoptosis process. Therefore, we treated rodents with lifetime intraperitoneal (I.P.) injection for 30 days to determine the potential therapeutic effect of insulin compared to control groups. The caspase cascade plays a crucial role in programmed cell death, as shown before. Autophagy and apoptosis pathways maintain cell survival (16-20). Two main pathways are accepted as activators of the caspase cascade in apoptosis: intrinsic and extrinsic pathways (17-19). Changes in mitochondrial permeability play a crucial role in the intrinsic pathway regulated by pro-apoptotic

and antiapoptotic proteins. When activated by apoptotic stimuli, pro-apoptotic factors like Bax and Bak affect the mitochondrial outer membrane permeability and lead to the activation of the caspase cascade. Bcl-2 is an antiapoptotic factor that frustrates Bax and Bak, activated by the PI-3K/Akt pathway (19,21). This pathway can also stimulate the expression of Foxo-1, which engages in cell proliferation (21). The insulin-like growth factor 1 (IGF-1) is a regulatory hormone that plays a crucial role in cell proliferation and growth (21-23). By binding to IGF-1 receptors (IGF-1R), this factor leads to the phosphorylation of insulin receptor substrates (IRSs) which can activate the PI-3K in the following (21-23). PI-3K blocks the effects of Bax through the PI-3K/AKT signaling pathway, as mentioned before. Following our study, previous research has shown that healthy longevity is associated with maintaining brain insulin function (1,2,24). Given the increasing prevalence of neurodegenerative diseases and cognitive disorders associated with aging, improving brain insulin function may be an essential treatment option to facilitate health in aging (1-5).

Conclusion

In conclusion, our result indicated that treating mice with insulin prevented reducing the number of neurons and gene expression related to normal brain function. Insulin may be beneficial as a new approach to avoiding neuron loss in regenerative medicine.

Acknowledgment

This study financially supported the Hearing Disorders Research Center, Loghman Hakim Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran (Registration No: 24901).

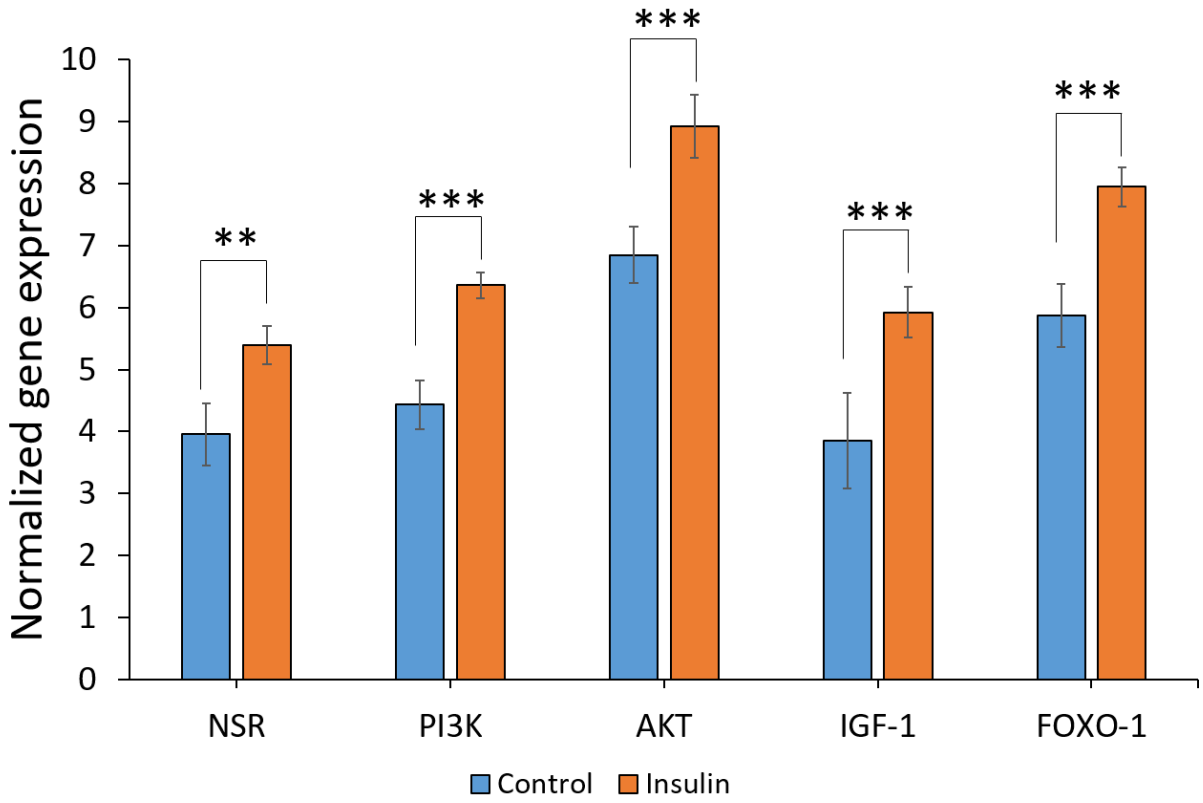

Figure 2. Real-time-PCR analyses. mRNA expression levels of (NSR, PI3K, AKT, IGF-1, and FOXO-1) in the brain tissue. Mean ± SD of the gene expression in the different groups. The significant difference between control with the insulin groups is indicated (**P < 0.01 and ***P < 0.001). PCR: Polymerase Chain Reaction.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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