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Effect of Mitochondrial Calcium Concentration in Regulating SynGAP by Influencing Mitochondrial Dynamics in HT22 Mice

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Authors' Contribution
DL and YG conceived the design of the study, collected and reviewed the data, and did coordination of project. YZ and WY did literature review, collected the data and prepared the manuscript. DX and WY helped in critical revision and finalizing the manuscript. All authors read, revised, and approved the final manuscript.

Key words
Mitochondrial calcium ion, Mitochondrial dynamics, Invasion, Migration, Apoptosis, SynGAP

ABSTRACT

The objective of this study was to observe the effect of mitochondrial calcium ion on the damage of hippocampal neurons in HT22 mice, so as to clarify the mechanism of mitochondrial calcium ion regulating synaptic GTPase activating protein (SynGAP) by influencing mitochondrial dynamics. The hippocampal neurons of HT22 mice were randomly divided into control group (50μl saline was added), low-dose experimental group (25μg Ca^{2+} 50μl), middle-dose experimental group (50μg Ca^{2+} 50μl) and high-dose experimental group (100μg Ca^{2+} 50μl), totally 4 groups. The cell death rate of hippocampal neurons in HT22 mice was detected by LDH release method. The production rate of mitochondrial ROS was detected by DCFH-DA fluorescent dye. Cell mitochondrial membrane potential was tested by flow cytometry. The expressions of SynGAP, OPA1, MFN1, SYN1 and BDNF in hippocampal neurons of HT22 mice were detected by Western blot with the increase of mitochondrial calcium ion concentration, the survival rate of HT22 cells increased, and the cell death rate of HT22 mice hippocampal neurons decreased significantly ($P<0.05$). Mitochondrial calcium ion treatment reduced intracellular ROS and mitochondrial membrane potential in a dose-dependent manner ($P<0.01$). In addition, the protein levels of SynGAP, OPA1, MFN1, SYN1 and BDNF in hippocampal neurons of HT22 mice were significantly increased after mitochondrial calcium ion treatment ($P<0.05$). It was concluded that mitochondrial calcium ion inhibits the death of hippocampal neurons in HT22 mice and promotes their survival. Mitochondrial calcium ions promote the expression of mitochondrial dynamics related proteins SynGAP, OPA1, MFN1, SYN1 and BDNF in hippocampal neurons of HT22 mice. Mitochondrial calcium can inhibit the death of hippocampal neurons by reducing the accumulation of ROS in hippocampal neurons.

INTRODUCTION

Mitochondria is a dynamic organelle, which participates in many cellular processes, such as ATP synthesis, calcium homeostasis and cell death (Arif *et al*., 2022). It increases its energy supply through continuous fusion and division (Rangaraju *et al*., 2019; Liang *et al*., 2021). Mitochondrial dynamics (mitochondrial fusion and division) is an important process to maintain normal morphology, quality and function of mitochondria. The energy supply of the brain mainly depends on the quality control of mitochondria, which play a momentous action in maintaining the metabolic homeostasis in the brain and regulating the energy supply, calcium ion release and neurotransmitter transport of neurons (Chen and Chan, 2009).

Neurodegenerative diseases (NDDs) are caused by gradual loss of neuronal structure and function or neuronal death (Walker *et al*., 2013; Rouault, 2013). The pathogenesis of most NDDs is still unclear, but more and more evidences show that the pathogenesis of neurodegenerative illness is related to mitochondrial dynamics (Chen and Chan, 2009). NDDs are a heterogeneous illness characterized by dysfunction or absence of neurons or myelin sheaths, which bring about dysfunction of the nervous system. The clinical

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Abbreviations used:
BDNF, brain-derived neurotrophic factor; SYN1, synapsin-I; Syn GAP, synaptic GTPase-activating protein; MFN1, mitofusin; OPA1, mitochondrial dynamin like GTPase

manifestations are cognitive dysfunction, motor dysfunction, aphasia, optic nerve damage and other symptoms. As a very common intracellular signal transmission system, calcium ions control many aspects of information transmission between neurons and glial cells. In neurons, calcium signaling system regulates the extracellular secretion of neurotransmitters, and establishes a connection between the excitement of postsynaptic cells and the integration of intracellular events, controlling synaptic strength, gene expression and memory function (Bezprozvanny, 2009). In order to better understand the mechanism of mitochondrial dynamics in NDDs, we studied the effect of mitochondrial calcium concentration on HT22 cells through mitochondrial dynamics. At present, there is no relational exploration on whether there is a correlation between the neuroprotective effect of mitochondrial calcium and mitochondrial dynamics. Therefore, in this experiment study, mitochondrial calcium ion was applied to hippocampal neurons of HT22 mice to observe its protective effect and further explore its possible framework, so as to supply new ideas and targets for the treatment of Alzheimer's disease.

MATERIALS AND METHODS

HT 22 cells

Mouse hippocampal neural precursor cell line HT22 (Guangdong Jinio Company, origin: USA) and 1 bottle of resuscitation cells (T25 culture bottle) were passed to the 4th generation. The cells were cultured in complete medium (420 mL DMEM medium (84%) +50 mL of FBS (10%) +5 mL of monoclonal antibody (1%)) and then divided into non-intervention group (50µl saline was added), low-dose experimental group (25µg Ca^2), middle-dose experimental group (50µg Ca^2) and high-dose experimental group (100µg Ca^2).

MTT assay

Single cell suspension was prepared with culture medium containing 10% fetal calf serum and inoculated into 96-well plate with 1000-10000 cells per well and 200µl volume per well. After 3-5 days of culture, each well was added with MTT solution (5mg/ml prepared with PBS, pH=7.4) 20µl and incubated for 4h. DMSO (150µl) was added to each well, and shaken for 10 min. The light absorption value of each well was measured on the enzyme-linked immunomonitor at 490nm.

Detection of cell death rate of hippocampal neurons

A background control group containing only DMEM culture solution was set up in the experiment. After the corresponding time of drug treatment, 20µl of LDH releasing reagent was extracted and added into the sample. The culture plate was incubated in a carbon dioxide incubator at 37°C for 1h. After 1h, culture plate was centrifuge at 1500 r/min for 5 min and then transferred to a new 96-well plate. 60µl of LDH reaction solution was added and then shaken at a medium speed at 25°C in the dark for 30 min. OD was taken at wavelength of 490nm with an enzyme-labeled instrument (HBS-1096A).

Detection of ROS production rate in mitochondria with DCFH-DA fluorescent dye

0.1mL of mitochondrial suspension was added with 2.9 mL of active oxygen measuring medium (1.5 mg defatted BSA, 1mmol/L EDTA, 130 mmol/L KCL, 2.5mmol/L KH2PO4, 10 mmol/L Hepes, pH7.4), 0.1 mmol/L malic acid +1 mmol/L glutamic acid to start the tetramitochondrial respiration, and 5 µ mol/L DCFH was added. The dynamic changes of fluorescence intensity were recorded by fluorescence spectrophotometer, and the calculated slope was the production rate of mitochondrial reactive oxygen species.

Detection of transmembrane potential of mitochondrial membrane in cells

Cationic fluorescent substance R123 can aggregate on active mitochondria, and its aggregation degree can reflect the transmembrane potential of mitochondria. After the cells were treated with the treatment solution, the cells were treated with 500 nmol/L R123 for 30min, incubated at 37°C, washed with buffer solution, and excited by 530 nm emission light with a wavelength of 488 nm. The intensity of excitation light reflected the transmembrane potential of mitochondrial membrane.

Quantitative reverse transcription PCR (qRT-PCR)

At specific time intervals during the experiment, samples were obtained, and RNA was isolated from the cells by an RNA extraction kit following the guidelines provided by the manufacturer (Thermo Fisher Technology China). RNA (1µg) was mixed with an appropriate quantity of RNase-free water, and proceeded for synthesis of cDNA by reverse transcription reaction in a thermal cycler. The prepared PCR product was visualized in agarose gel after the electrophoresis to confirm the validity of the samples.

Western blot

The cells to be tested were washed with precooled PBS solution 150µl precooled RIPA (Radio Immunoprecipition Assay) lysate (40µl of 25× protease inhibitor and 100µl of 10× phosphatase inhibitor was added to 1 mL of lysate), ice-bathed for 30 min, and samples were collected with a spatula, and centrifuged at 11500 rpm at 4°C for 15 min. The cell lysate was boiled and denatured after adding

loading buffer. It was then subjected to 8% SDS-PAGE. The protein pattern was transferred to a membrane which was immersed in TBST solution containing 5% skim milk (20 mmol/L Tris-HCl, PH 7.14, 150 mmol/L NaCl, 0.1%Tween20) and incubated at room temperature for 60 min. After adding primary antibody (rabbit anti-p65 and anti-phosphorylated pp65, 1:1000; rabbit anti-p38 and pp38, 1:1000; mouse anti-TLR9, 1:1000; rabbit anti-podocin, 1:1000; rabbit anti-CD2AP, 1:1000), HRP labeled goat anti-rabbit secondary antibody (1-15 000) was added and incubated at room temperature for 1 hour. After being washed by TBST, the color was developed by enhanced chemiluminescence system (ECL, Millipore), and the absorbance of protein bands was photographed and scanned in GIS gel image analysis system and analyzed.

Statistical analysis

The data was analyzed by SPSS16.0 software and presented as the mean±SD. The distinction between the two groups was examined using a t-test, while the variation among multiple groups was analyzed through one-way ANOVA, followed by the implementation of the LSD test. A P-value less than 0.05 was deemed to be statistically significant.

RESULTS

The activity of hippocampal neurons in HT22 mice was significantly raised in the middle-dose experimental group and the high-dose experimental group after treatment compared to control group ($P<0.05$), suggesting that the concentration of mitochondrial calcium ion can increase the activity of hippocampal neurons in HT22 mice (Fig. 1A). The death rate of hippocampal neurons in HT22 mice was significantly reduced in the middle-dose experimental group and the high-dose experimental group after treatment than control group ($P<0.01$), suggesting that the concentration of mitochondrial calcium ion can reduce the death rate of hippocampal neurons in HT22 mice (Fig. 1B).

The levels of SynGAP in hippocampal neurons of HT22 mice were gradually increased after the treatment with Ca^{++} ions compared with the control group ($P<0.01$), suggesting that the concentration of mitochondrial calcium ion can activate the expression of SynGAP in hippocampal neurons of HT22 mice (Fig. 2).

Figure 3 shows that the protein levels of OPA1 and MFN1 in hippocampal neurons of HT22 mice increased gradually than control group ($P<0.01$), suggesting that the concentration of mitochondrial calcium ion can activate the expression of OPA1 and MFN1 in hippocampal neurons of HT22 mice (Fig. 3).

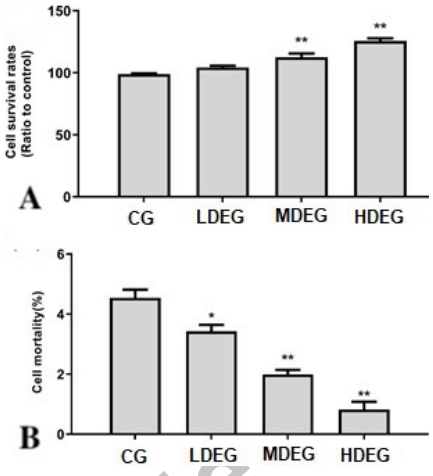

Fig. 1. Effect of mitochondrial calcium ion concentration on survival activity and detection of hippocampal neurons. CG, control group; LDEG, low dose experimental group; MDEG, medium dose experimental group; HDEG, high dose experimental group.

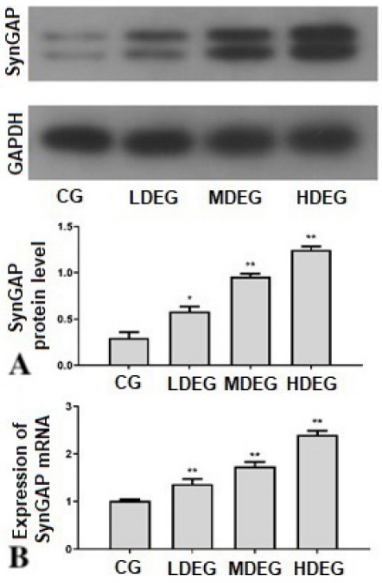

Fig. 2. Effect of mitochondrial calcium ion concentration on the expression level of synaptic GTPase activating protein (SynGAP). (A), Tested by Western blot; (B), Detected by qRT-PCR.
For abbreviations, see Figure 1.

In order to observe whether the concentration of mitochondrial calcium ion causes mitochondrial excitability, it was found that mitochondrial calcium ion can significantly reduce the release of ROS in cells. The release of ROS in cells in the middle dose experimental

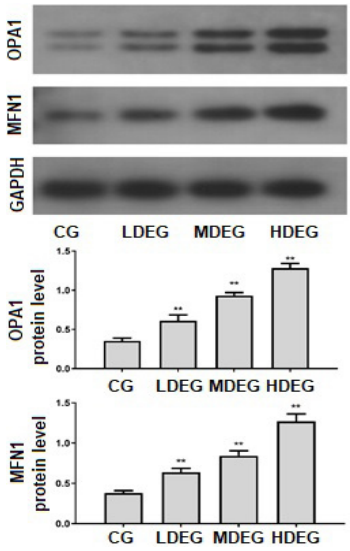

Fig. 3. Effect of mitochondrial calcium concentration on optic atrophy (OPA1) and Mitofusin-1 (MFN1) in hippocampal neurons of HT22 mice.
For abbreviations, see Figure 1.

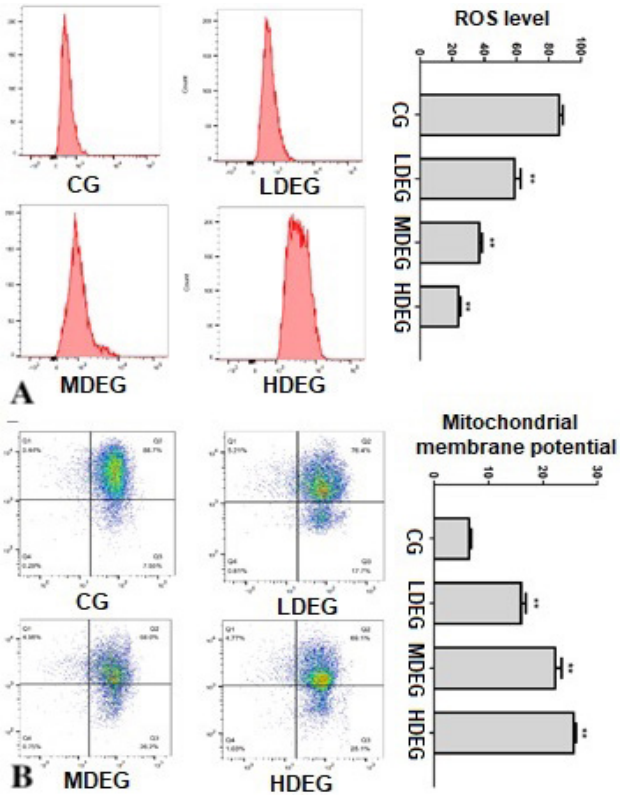

Fig. 4. Effect of mitochondrial calcium concentration on release of intracellular ROS (A) and on cell mitochondrial membrane potential (B) by flow cytometry.
For abbreviations, see Figure 1.

group and the high dose experimental group can be reduced in a dose-dependent manner than control group ($P<0.01$), evidence that the change of intracellular oxidative stress level was related to stimulating mitochondrial excitability. Mitochondrial calcium ions significantly increased the mitochondrial membrane potential of cells. The middle-dose experimental group and the high-dose experimental group significantly increased the mitochondrial membrane potential than control group ($P<0.01$) (Fig. 4).

The protein levels of SYN1 and BDNF in hippocampal neurons of HT22 mice were gradually increased after treatment in middle-dose experimental group and high-dose experimental group than the control group ($P<0.01$), suggesting that the concentration of mitochondrial calcium ion can activate the expression of SYN1 and BDNF in hippocampal neurons of HT22 mice (Fig. 5).

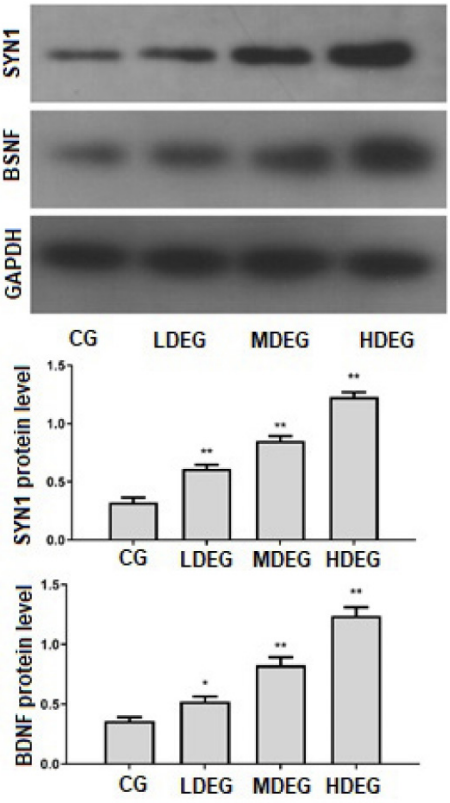

Fig. 5. Effect of mitochondrial calcium ion concentration on the protein levels of SYN1 (aSyn) and BDNF in hippocampal neurons of HT22 mice.
For abbreviations, see Figure 1.

The results of immunofluorescence were consistent with those of Western blot. The number of BDNF-positive cells in the hippocampus of HT22 mice was significantly increased in the middle-dose experimental group and the

high-dose experimental group than control group (P<0.05) (Fig. 6).

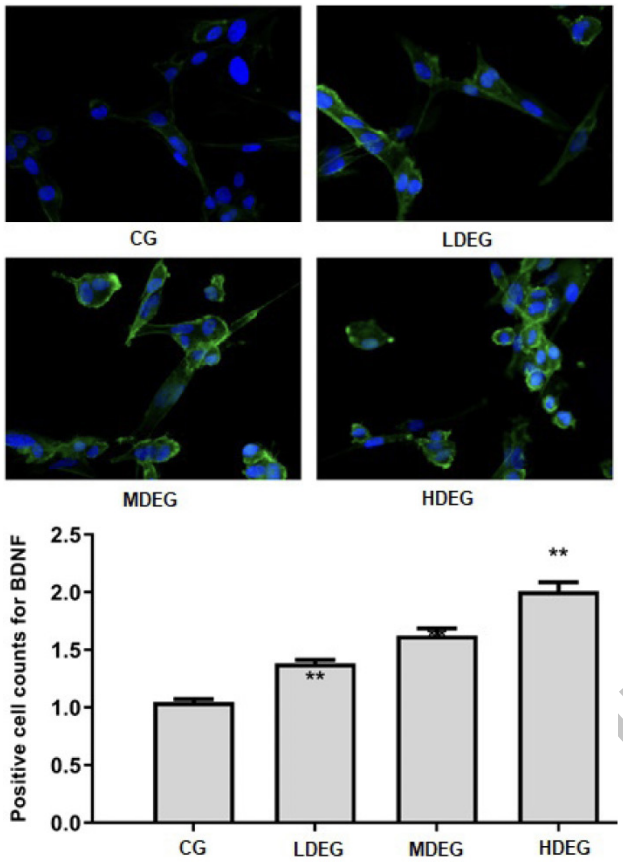

Fig. 6. Effect of mitochondrial calcium ion concentration on BDNF fluorescence intensity.
For abbreviations, see Figure 1.

DISCUSSION

HT22 mouse hippocampal neuron cell line is a good model to study neurotoxicity in vitro, which is extensive used in the study of cranial nerve illness (Jia *et al*., 2014; Lee *et al*., 2018). The results of this study show that mitochondrial calcium ions inhibited the death of hippocampal neurons in HT22 mice and promoted cell survival.

SynGAP is a kind of Ras GTP enzyme activating protein (Ras GAP) located in synapses, which is selectively expressed in the brain, especially in excitatory synapses (Hamdan *et al*., 2019). Recently, it has been found that SynGAP is the main catalytic substrate of CAMK ii (Guo *et al*., 2009). After SynGAP is phosphorylated by CAMK ii, its Ras GTP enzyme activity is inhibited (Penda *et al*., 2008). The study showed that SynGAP in hippocampal neurons of HT22 mice increased after treatment with mitochondrial calcium ion, and it was dependent on the concentration of mitochondrial calcium ion.

Mitochondria are organelles that are in a highly mobile state and frequently fuse and divide (Narendra *et al*., 2010). Under physiological conditions, the fusion and division of mitochondria are in a state of balance, which is regulated by MFN1 and OPA1 (Song *et al*., 2009). MFN1 mediates the fusion of mitochondrial outer membrane (Maremanda *et al*., 2021). These proteins are regulated by ubiquitination and proteolysis. For neurons, the dynamic balance of mitochondrion fusion and division is very important to ensure the long-distance transportation and average energy distribution of neuron endings. Therefore, abnormal mitochondrion fusion and division may be one of the pathogenic factors of many neurodegenerative diseases (Kar *et al*., 2010). The results found that the levels of MFN1 and OPA1 in hippocampal neurons of HT22 mice increased after treatment with mitochondrial calcium ion, which was dependent on the concentration of mitochondrial calcium ion.

Among many factors that cause intracellular DNA damage, the increase of intracellular ROS is the most interesting DNA damage factor (Chompoosor *et al*., 2010). Under physiological conditions, ROS plays an momentous role in cell signal transduction and maintaining the stability of the body (Bernstein *et al*., 2011). When organisms are stimulated by pathogens, calcium, radiation and other internal and external environments, the balance between oxidation and antioxidant systems is out of balance, which leads to a large number of ROS production and accumulation in cells, causing oxidative stress (Cemeli, 2011). ROS can produce many forms of DNA damage, such as base mutation, DNA strand breakage, DNA strand abnormality, etc. (Tasdogan *et al*., 2016). At the same time, DNA damage can also induce oxidative stress in the body, which will lead to cell death (Mittal and Pandey, 2014). Brain tissue consumes the most oxygen in all tissues and organs of human body, and neurons carry a large number of mitochondria. As early as 50 years ago, it was found that the respiratory chain of mitochondria would produce ROS. The increase of ROS can cause obvious DNA oxidative damage and nerve cell death (Newington *et al*., 2011). The results of this study showed that ROS in hippocampal neurons of HT22 mice decreased after mitochondrial calcium ion treatment, and it was dependent on the concentration of mitochondrial calcium ion.

Hippocampus is the key structure of learning and memory, and it is also the brain region with the richest expression of BDNF and its receptor TRK B (Broad *et al*., 2002). Long-term potentiation (LTP) is closely

related to learning and memory. At present, a large number of experiments have confirmed that BDNF plays a momentous effect in the induction and maintenance of LTP and hippocampus-dependent learning and memory (Kim *et al*., 2013). At the same time, BDNF can also regulate the shape of synapse, increase axon bifurcation, grow dendrites and enhance synaptic function (Shen *et al*., 2016). The complex of BDNF and its receptor can be transported to synapses, regulating the plasticity of synapses and the release of neurotransmitters (Lee *et al*., 2013). SYN is involved in the operation of vesicles and the release of neurotransmitters. The increase of SYN means the enhancement of synaptic vesicles transport capacity and transmission efficiency, which indicates that mitochondrial calcium ions can affect the synaptic plasticity of SYN1 and BDNF genes.

CONCLUSION

Mitochondrial calcium ion can inhibit the death of hippocampal neurons in HT22 mice and promote their survival. Mitochondrial calcium ions promote the expression of mitochondrial dynamics related proteins SynGAP, OPA1, MFN1, SYN1 and BDNF in hippocampal neurons of HT22 mice. Mitochondrial calcium can inhibit the death of hippocampal neurons by reducing the accumulation of ROS in hippocampal neurons.

DECLARATIONS

Acknowledgments

Thanks to the members from The Second Hospital of Tianjin Medical University, the group collected samples, obtained data, and theoretical guidance.

Ethical approval

The study was carried out in compliance with guidelines issued by Ethical Review Board Committee of The Second Hospital of Tianjin Medical University, China. The official letter would be available on fair request to corresponding author.

Statement of conflict of interest

The authors have declared no conflict of interest.

IRB approval

This study was approved by the Advanced Studies Research Board of the Second Hospital of Tianjin Medical University, Tianjin, 300211, China.

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