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Mitophagy in APPsw/PS1dE9 transgenic mice and APPsw stably expressing in HEK293 cells

X. WANG[1], X.-L. ZHAO[1], L.-L. XU[1], C.-F. WANG[1], L.-F. WEI[1], Z. LIU[1], H. YANG[1], P. WANG[1], Z.-H. XIE[1], J.-Z. BI[1,2,3]

[1]Department of Neural Medicine, Second Hospital of Shandong University, Jinan, China
[2]Institute of Neurology, Shandong University, Jinan, China
[3]Key Laboratory of Translational Medicine on Neurological Degenerative Disease, Shandong University, Jinan, China

Abstract. – OBJECTIVE: To observe mitophagy in APPsw/PS1dE9 transgenic mice and APPsw stably expressing HEK293 cell.

MATERIALS AND METHODS: The APPsw /PS1dE9 transgenic mice and 20E2 cell (HEK293 cell with APPsw) was used to be the model of Alzheimer's disease. We dynamically observed the behavior, mitochondrial structure and mitophagy in brain of APP/PS1 transgenic mice in different age groups. Mitochondrial structure and mitophagy of HEK293 and 20E2 cell *in vitro* were also recorded.

RESULTS: The mitochondrion was changed significantly in APP/PS1 transgenic mice and 20E2 cell. LC3 protein, associated with autophagy, was up-regulated. Mitophagy related protein PINK1 and PARKIN were up-regulated.

CONCLUSIONS: Mitophagy disorder may be associated with the pathogenesis of Alzheimer's Disease through PINK1/Parkin pathway.

Key Words:
APP/PS1 mouse, Alzheimer's disease, Mitophagy, PINK1, Parkin.

Introduction

Mitochondrion is recognized as the energy provider in eukaryotic cell activity. As reactive oxygen species (ROS) producer, it has a high prevalence of ROS damage and disorders [1], which may cause pathological changes[2]. Mitophagy is that dysfunctional mitochondrion needs to be removed timely to protect the whole cell and be selective eliminated through autophagy/lysosomal pathway[3,4]. Mitophagy interrupts the mitochondrion-dependent apoptosis, which causes dysfunctional mitochondrion aggregation and neurodegenerative disease further.

PINK1/Parkin pathway in mitophagy includes two recessive linked gene of Parkinson's disease: PTEN induced putative kinase 1 (PINK1) and E3 ubiquitin ligase Parkin[5-7]. In normal mitochondrion, as the inhibited membrane voltage, full-length form of PINK1 experiences proteolysis. After the depolarization of membrane voltage, proteolysis is inhibited. Then, PINKl can be stable on mitochondrial outer membrane[8,9]. PINK1, located in upstream of Parkin, phosphorylates Parkin, which plays an important role in mitophagy[10-12]. The interaction between PINK1 and Parkin improves ubiquitination of mitochondrial outer membrane protein and induces mitophagy.

In neurodegenerative diseases, mitophagy disorder was usually found in many studies. In the early stage of Alzheimer's Disease (AD), mitophagy disorder accelerates reactive oxygen species (ROS)[13]. Meanwhile, the division and fusion of mitochondrion are changed. And the weakened cytolysosome reduces mitophagy[14]. But, Moreira et al[15] found that mitophagy was enhanced in AD cell. In this study, we used the APPsw/PS1dE9 transgenic mice[16] and 20E2 cell (HEK293 cell with APPsw) to be the model of AD[17,18]. Then, mitochondrial structure and mitophagy related proteins were observed.

Materials and Methods

Animals and Tissues

We used the APPsw/PS1dE9 transgenic C57BL/6 mice at 3rd, 6th, 9th, 12th month old. 8-9 month-old C57BL/6 mice were control. All mice were male (n=15 per group). All mice were from Beijing HFK Bioscience Co., Ltd. Hippocampus was obtained and stored at –80°C. The animals

Corresponding Author: Jianzhong Bi, MD; e-mail: bjz@sdu.edu.cn
Zhaohong Xie, MD; e-mail: xie_zhaohong@126.com

were used following the guidelines of the Ethical Committee for Animal Experiments of Shandong University.

Cell Culture and Treatment

HEK293 cell and 20E2 cell (HEK293 cell with APPsw)[17] were donated by Professor Xiulian Sun in Qilu Hospital of Shandong University. They were cultured in 37°C, 5% CO_2 and DMEM medium (HyClone Co., South Logan, VT, USA) with 10% fetal calf serum (Gibco Co., Grand Island, NY, USA). 6 μl 100 mg/ml G418 solution (Sigma Co., St. Louis, MO, USA) was added per 6 ml medium. Passage per 2-3 days. After washed by phosphate buffered saline (PBS) twice and treated by 0.25% trypsin (HyClone Co., South Logan, VT, USA), cell was observed under the microscope.

Behavior test

Learning and memory ability was evaluated by Morris water maze. The whole process in-

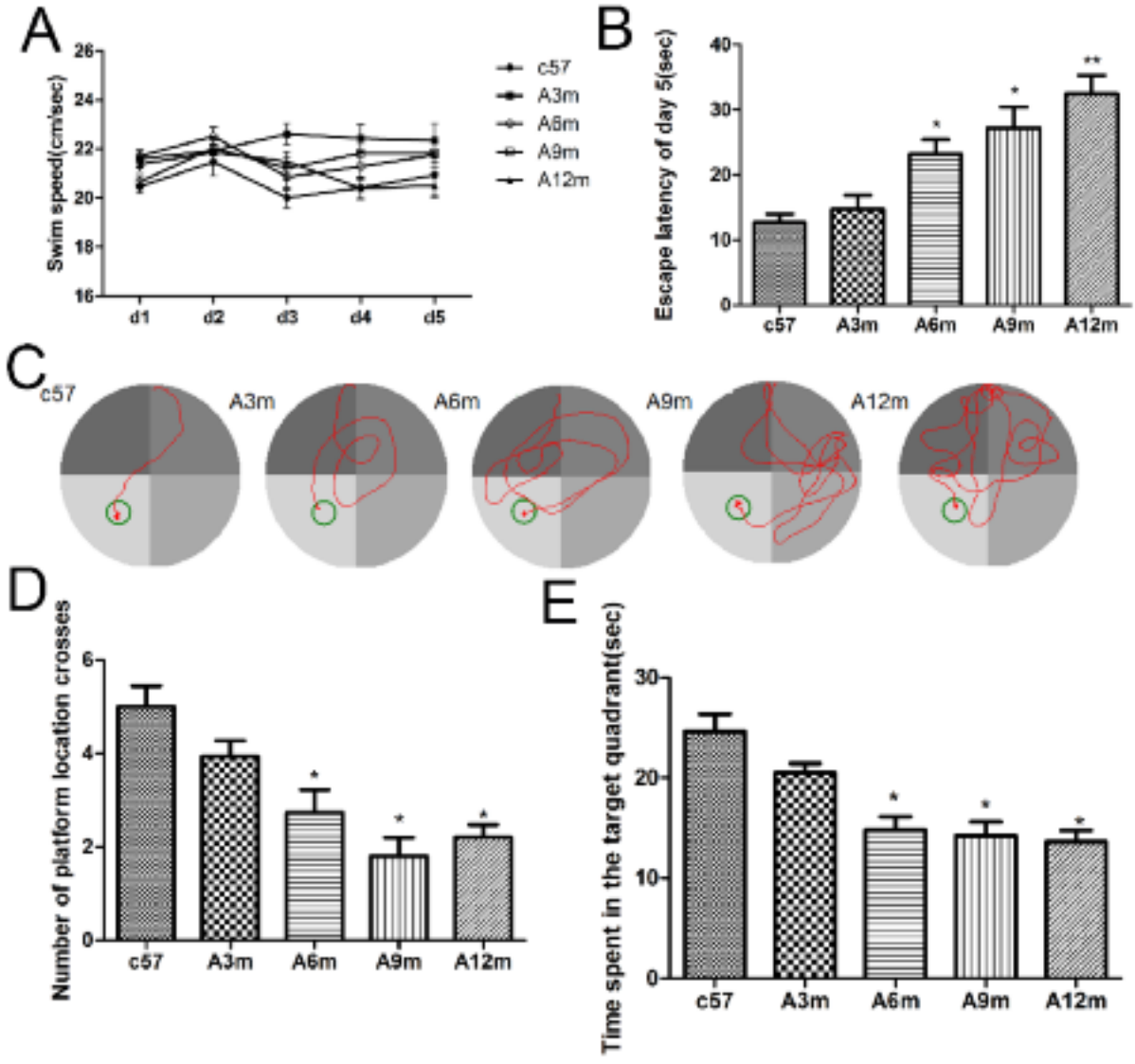

Figure 1. Behavior changes. The comparison of latent time, the time and times of getting old goal location, swimming speed in transgenic mice and control. ***A)*** There was no significant difference between groups in swimming speed. ***B)*** After 5 days train, the latent time of transgenic mice above 6 month was considerably reduced, compared to control. And this gap was largest in the 12 month group (*$p < 0.05$, **$p < 0.01$, n = 15). There was no significant difference for the 3 month group. ***C)*** The pathway map to old goal location at 5th day. Control tended to the old goal, while the transgenic mice above 6 month was randomly to the goal. ***D-E)*** The time and times of getting old goal location in transgenic mice above 6th month was significantly different with control (*$p < 0.05$, n = 15). A = APPsw/PS1dE9; m = month

cluded 1 day adaptive phase without platform, 5 days learning phase with hiding platform, and 1 day exploratory phase after 24h of the last learning phase[19]. At learning phase, the longest time of finding the platform was 60s. If a mouse failed to arrive in time, it would be guided to the platform. Every mouse was trained 4 times per day. The platform was located at SW. Start locations were randomly divided at N, E, SE, NW. The mouse explored and arrived at the platform was described as latent time. After 24h of the last learning experiment, the platform was removed to perform exploratory experiment to test memory ability. And NE was the new start location. The experiment time was 60s. In the exploratory experiment, the time and times of getting old goal location were recorded to evaluate the learning ability.

Electron Microscopy (EM)

The brain tissue and cell were fixed in ice-cold glutaraldehyde (3%, pH=7.4). After washed by PBS, it was fixed by osmic acid (OsO4) and embedded in EPON812 resin. Then it was cut successively to 0.06 μm chips. Uranyl and lead citrate were used to stain. And it was observed by JEM-1200 EX electron microscope.

Western Blotting

Tissues and cells were digested by radioimmunoprecipitation assay (RIPA) (25 mM Tris-HCl, pH 7.6; 150 mM NaCl; 1% NP40; 1% sodium deoxycholate; 0.1% sodium dodecyl sulfate, SDS) in cold for 30 min. Concentration of protein was tested by bicinchoninic acid (BCA) method. Denaturation in binding buffer at 100°C for 5 min. 12% separation gel and 5% stacking gel were prepared. SDS-PAGE electrophoresis (140V) was performed. The protein was transported to the polyvinylidene fluoride (PVDF) membrane (150 mA, constant-current, 2h). Block the membrane with 5% skim milk in tris buffered saline/t (TBS/t) solution for 1 hour. Add primary antibody and incubate overnight in 4°C. Wash the membrane with TBST for 3 times (5 min per time). Add secondary antibody and incubate for 1 hour. After wash the membrane, the protein was exposed by electrochemiluminescence (ECL).

Antibodies

The antibodies for the Western blot analysis were LC3B (Sigma, St. Louis, Mo, USA, L7543, 1:3000); anti-PINK1 (Abcam, Cambridge, MA, USA, ab75487, 1:500), anti-Parkin (Cell Signaling Technology, Danvers, MA, USA, 4211, 1:1000), anti-β-actin (Proteintech, 66009-1-Ig, 1:5000).

Statistical Analysis

Data are presented as mean ± standard deviation ($x \pm$ SEM) and were analyzed using SPSS17.0 (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was considered as statistically significant difference. The data of Morris water maze used analysis of variance (ANOVA)[20]. Two variables used two-factor variance analysis. The comparison among groups was analyzed by single factor variance analysis. The comparison of mean used Least Significant Difference (LSD) post analysis. $p< 0.05$ was considered statistically significant.

Results

Behavior Change

Latent time, the time and times of getting old goal location were associated with not only distance the mouse traveled but also swimming speed in Morris water maze results. So, we evaluated the swimming speed first, and found no significant difference (Figure 1A). But, the learning ability of APP/PS1 transgenic mice was considerably lower than C57 mice. In learning experiment, the latent time was longer than control in 6 month group. With the increase of age group, the latent time was increasingly longer, which was more evident at 5th day (Figure 1 B,C). The memory ability of APP/PS1 transgenic mice was also considerably lower than C57 mice. In the exploratory experiment, the time and times of getting old goal location were longer than control, and the gap was enhanced with the increase of age group (Figure 1 D,E). So, the cognition of 6 month-old APP/PS1 transgenic mice was changed. And their learning and memory ability were remarkably reduced, which was more enhanced with the increase of age.

Ultrastructure Changes in Brain Tissue

Transmission electron micros was used to observe the hippocampus CA3 zone ultrastructure of APP/PS1 transgenic and C57 mice at 6, 12 month-old. In Figure 2, the brain tissue in APP/PS1 transgenic mice had many damaged degenerative mitochondrion and intramedullary lesions with many autophagic vacuoles. And these changes were greater with the increase of age and similar in both frontal cortex and hippocampus.

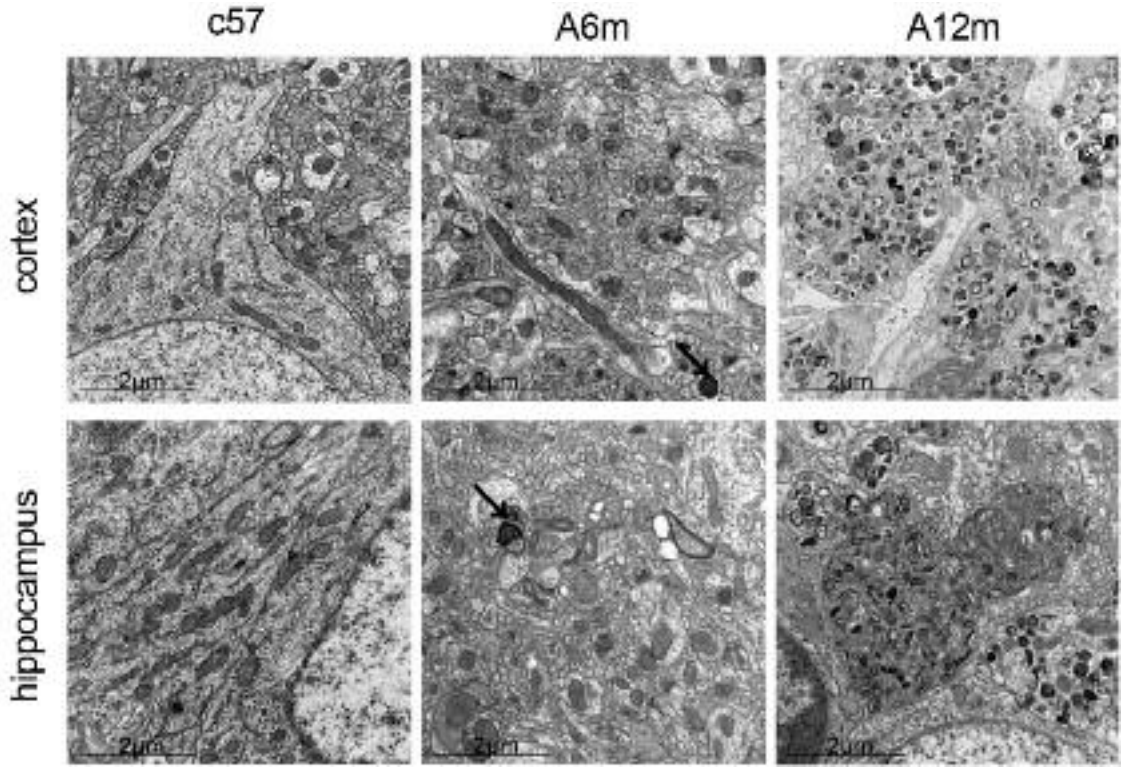

Figure 2. Ultrastructure changes of brain tissue. We observed frontal cortex and hippocampus CA3 zone ultrastructure of C57 mice at 8-9 month-old. The membrane structure of neuron axon and mitochondrial cristae were clear without many autophagic vacuoles. Hippocampus CA3 zone of APP/PS1 transgenic mice at 6 month-old had many damaged degenerative mitochondrion and intramedullary lesions with many autophagic vacuoles. And these changes were clearer in 12 month old group. A = APPsw/PS1dE9; m = month.

Expression level of LC3-II/LC3-I, PINK-1, Parkin in the Brain (Figure 3)

In Western blot results, autophagy markers LC3-II/LC3-I were up-regulated in 6 month-old APP/PS1 transgenic mice, compared to control. But, there was no significantly difference between 6 and 9, 12 month-old groups. For 3 month-old group, there was no significantly difference between transgenic mice and control (Figure 3B). PINK1 and Parkin were up-regulated in APP/PS1 transgenic mice, compared to control. And there was no significantly difference among different age groups (Figure 3C,D).

Mitochondrial change

In Figure 4, 20E2 cells had mitochondrial swelling, disappeared cristae, vacuolus, compared to HEK293 control.

Expression level of LC3-II/LC3-I, PINK-1, Parkin in 20E2 and HEK293 cell

Level of LC3-II/LC3-I, PINK-1, Parkin was enhanced in 20E2 cell than that in HEK293 cell, which indicated that mitophagy level was enhanced in 20E2 cell (Figure 5).

Discussion

APP and PS1 mutation in the same chromatosome, associated with familial AD, can cause age dependent amyloid plaque. In this study, praxeology of APP/PS1 transgenic mice was changed significantly. With the increase of age, memory ability was reduced. As is researched[21], autophagy took part in the pathogenic process of AD. Ratios of LC3II/LC3I was positively associated with quantity of autophagic vacuole, which was used to test autophagy level. In this study, the autophagy level of APP/PS1 transgenic mice was increased, especially in 6 month-old group. The autophagy level in 20E2 cell was also up-regulated, compared to HEK293 cell. So, our study confirmed this theory.

Free radical and oxidative stress are widely considered to take part in the pathogenic process

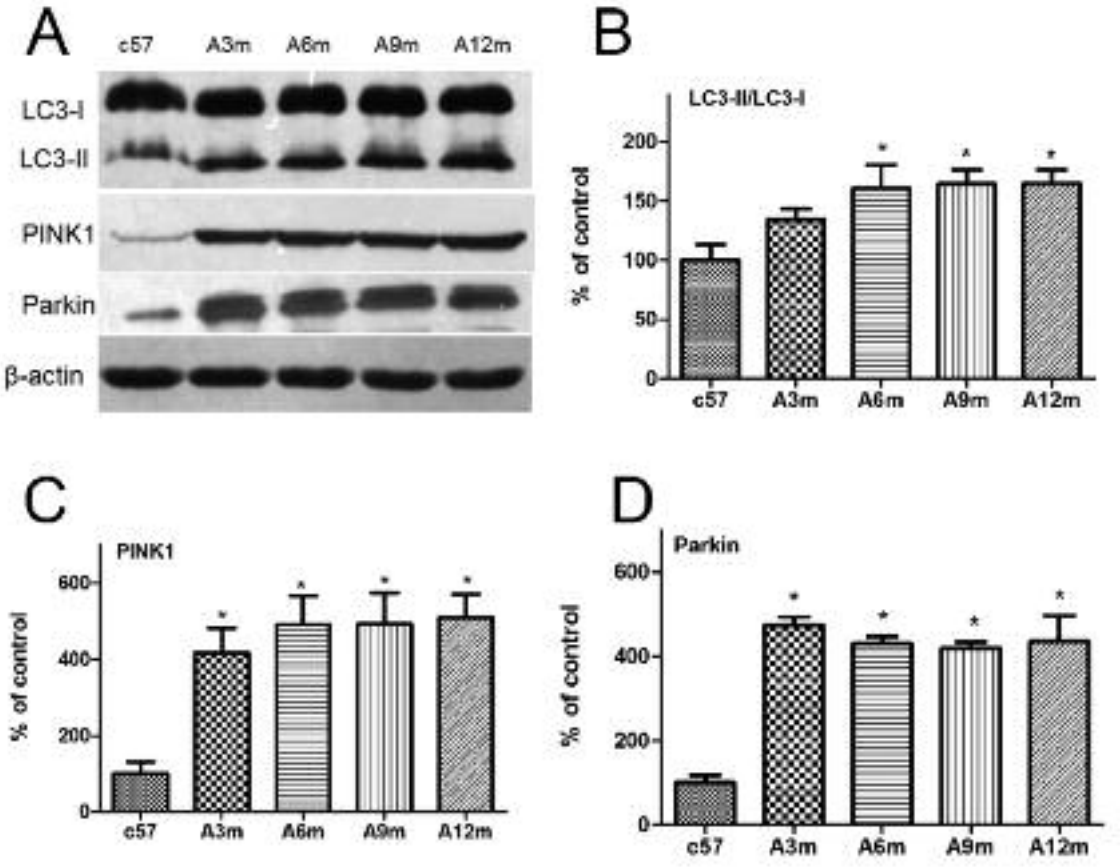

Figure 3. Expression level of LC3-II/LC3-I, PINK-1, Parkin in the transgenic mice were enhanced, compared to C57 mice. Internal parameter was β-Actin. ***A)*** Western blot results of LC3-II/LC3-I, PINK-1, Parkin. ***B)*** LC3-II/LC3-I level was to evaluate autophagy. For 3 month-old group, there was no significantly difference between transgenic mice and control. For 6, 9, 12 month-old group, LC3-II/LC3-I level in transgenic mice was significantly increased than control (*$p < 0.05$). There was no significantly difference among different age groups. ***C-D)*** There was significantly difference for PINK1 and Parkin level between different age groups and control (*$p < 0.05$). And there was no significantly difference among different age groups. A = APPsw/PS1dE9; m = month

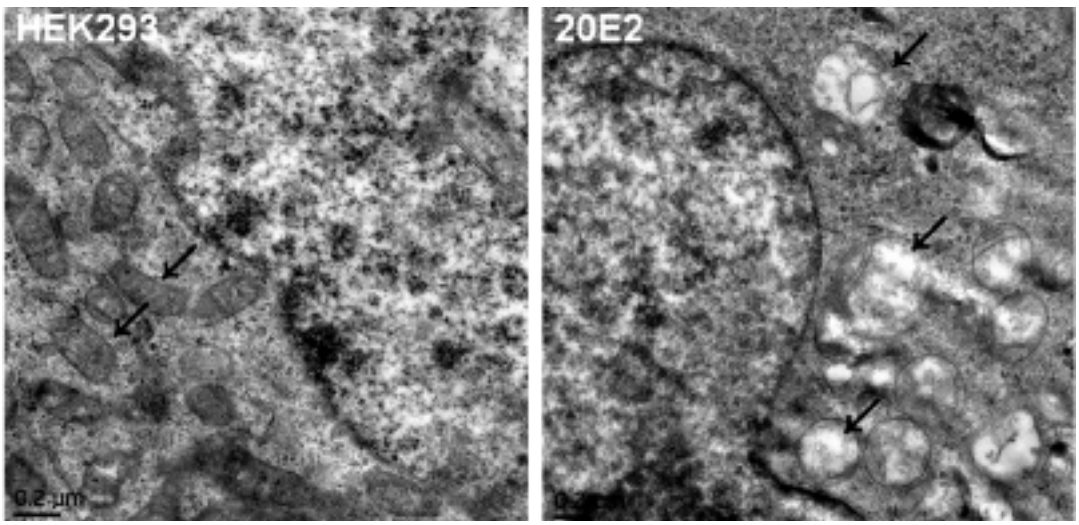

Figure 4. Mitochondrial changes. In HEK293 control, there were many normal mitochondrion. In 20E2 cells, mitochondrion structure was damaged (mitochondrial swelling, disappeared cristae, vacuolus).

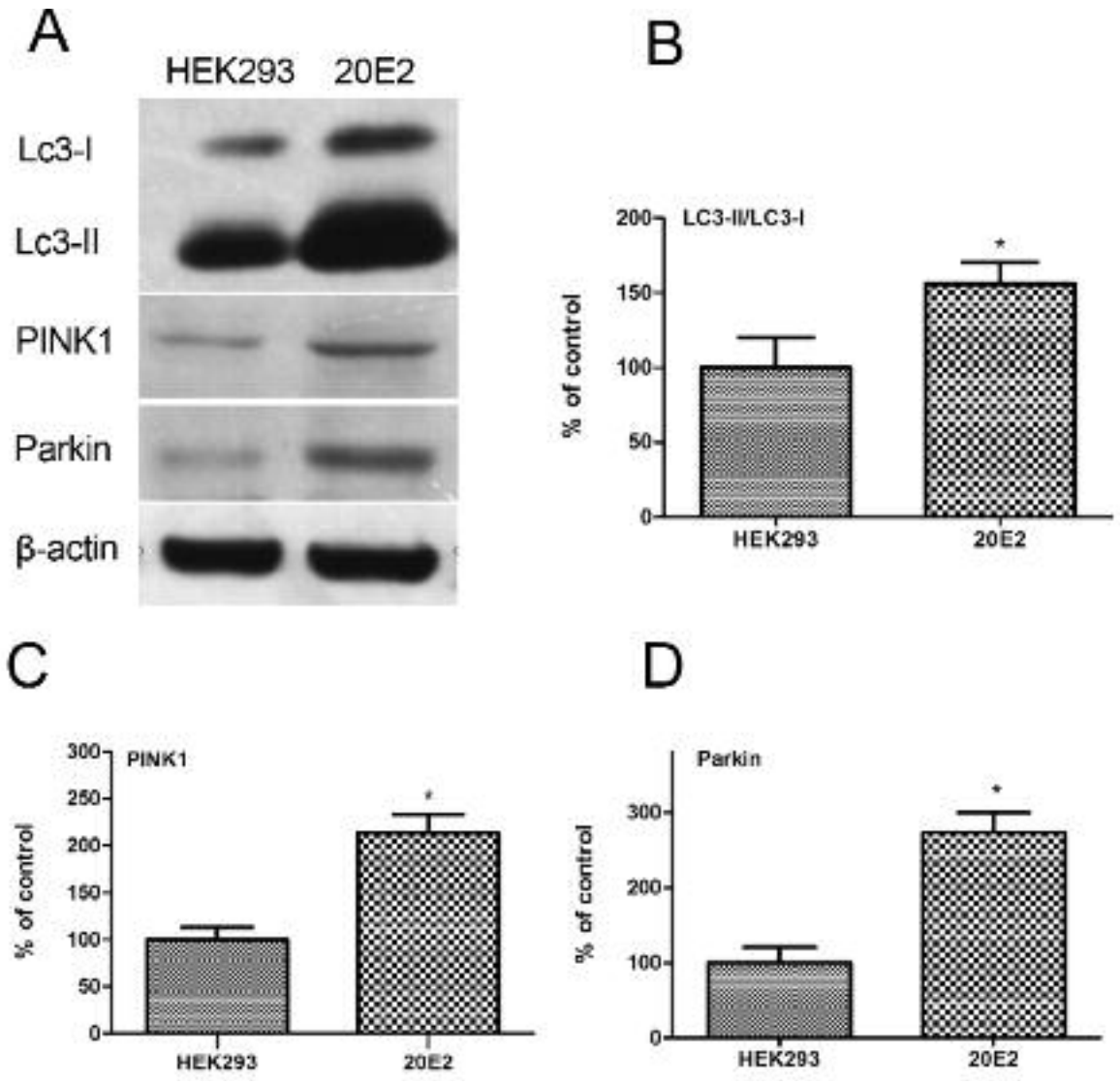

Figure 5. ***A)*** LC3-I, LC3-II, PINK-1, Parkin Level in HEK293 and 20E2 cell. ***B-D)*** Quantitive analysis of LC3-I, LC3-II, PINK-1, Parkin Level. Internal parameter was β-Actin. Level of LC3-II/LC3-I, PINK-1, Parkin was enhanced in 20E2 cell than that in HEK293 cell (*$p < 0.05$).

of neurodegenerative diseases, where mitochondrial metabolism plays a key role. Studies[22] showed that mitochondrial disorders pathological process of neuronal degeneration. Swerdlow et al[23] pointed mitochondrial cascade hypothesis, and reported that mitochondrion participated in the initiating process of AD, which was associated with Aβ deposition and tau protein. Aβ exists in mitochondrial matrix, inner and outer membrane, and can also interact with Amyloid-β Binding Alcohol Dehydrogenase (ABAD) and Cyclophilin D (CypD) to induce oxidative stress and ROS aggression. This process will damage mitochondrion, including decreased mitochondrion, membrane potential, ATP production, and axoplasmic transport disorder. Finally, denaturalized synapse and neuron weakened cognition. Hirai et al[24] found that the mitochondrion amount of hippocampus neuron in AD patients was decreased. Mitochondrion with damaged cristae and mitochondrial fragments were scattered in endochylema. In this study, we also confirmed the mitochondrial changes in APP/PS1 transgenic mice and 20E2 cell.

PINK1/Parkin pathway is the key factor associated with autophagy and abnormal mitochondrion elimination. When mitochondrion is damaged, PINK1 will stimulate Parkin move into mitochondrion to improve mitophagy. The mi-

tophagy change in brain of AD patients is still unclear. Currently, it is widely believed that mitochondrial autophagosome without available degradation is abundant in the brain of AD patients, which causes mitophagy activity lower[25]. However, Khandelwal et al[26] introduced Parkin into AD transgenic mice by lentivirus. It showed that mitophagy activity and abnormal mitochondrion elimination were enhanced. Shaerzadeh et al[27] injected Aβ protein into hippocampus CA1 domain in rats as the AD model, and found that the level of mitophagy markers was remarkably increased, indicating that mitophagy enhanced in the period of onset. In this study, the analysis of PINK1 and Parkin level also confirmed it.

Conclusions

Mitophagy enhanced, mitochondrial appearance changed, PINK1/Parkin level up-regulated in AD model. And it indicated that enhanced mitophagy might be mediated by PINK1/Parkin pathway.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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