LMTK2 inhibits Aβ$_{25-35}$-elicited ferroptosis, oxidative stress and apoptotic damage in PC12 cells through activating Nrf2/ARE signalling pathway

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Abstract

Alzheimer's disease (AD), the most common contributor to dementia, is a growing global health problem. This study aimed to investigate the role of lemur tyrosine kinase 2 (LMTK2) in AD as well as its relevant mechanism. To establish an in vitro cell model, PC12 cells were challenged with 20 µmol/l Aβ$_{25-35}$ for 24 h. RT-qPCR and western blot examined LMTK2 mRNA and protein expressions. With the application of CCK-8, TUNEL, iron colorimetric assay kit and DCFH-DA, the viability, apoptosis, Fe^{2+} and ROS content in PC12 cells were assessed. Besides, the expressions of oxidative stress-, apoptosis-, ferroptosis- and Nrf2/ARE signalling-related proteins were evaluated with western blot. Moreover, commercial kits examined SOD, MDA and CAT contents. The results manifested that LMTK2 expression was noticeably downregulated in Aβ$_{25-35}$-treated PC12 cells. Notably, LMTK2 overexpression exhibited inhibitory effects on oxidative stress, apoptosis and ferroptosis in PC12 cells exposed to Aβ$_{25-35}$. The upregulated Nrf2, NQO1 and HO-1 expressions in LMTK2 overexpressed-PC12 cells with Aβ$_{25-35}$ induction revealed that LMTK2 overexpression could activate the Nrf2/ARE signalling pathway. What is more, a series of cellular experiments further testified that ML385, a specific Nrf2 inhibitor, partly hindered the protective role of LMTK2 overexpression against Aβ$_{25-35}$-triggered oxidative stress, apoptosis and ferroptosis in PC12 cells. In conclusion, LMTK2 overexpression alleviated the ferroptosis, oxidant damage and apoptosis in PC12 cells exposed to Aβ$_{25-35}$ through the activation of the Nrf2/ARE signalling pathway, indicating the potential target of LMTK2 in the treatment of AD.

Key words: *Alzheimer's disease, LMTK2, ferroptosis, oxidative stress, apoptotic damage, Nrf2/ARE signalling pathway.*

Introduction

As a degenerative brain disease, Alzheimer's disease (AD) is the most prevalent contributor to dementia, making up 80% of all dementia cases and making an impact on more than 47 million people globally [5,9,22]. Dementia features declined capabilities in memory, language, problem-solving as well as other cognitive skills, the occurrence of which has a close relation with the damaged or destroyed nerve cells in parts of the brain implicated in cognitive function [2]. It was reported that age is the biggest contributor to

AD and the high incidence of AD is a result of the aging of the global population [2,21]. In addition, genetic factors, insulin hypertension, family history, unhealthy diet, and preventable head injury can also result in the occurrence of AD [2]. As far as we are concerned, there is no cure for this disease, and in view of this, more efforts are needed to be made to identify potential therapeutic targets.

Lemur tyrosine kinase 2 (LMTK2), which belongs to the family of membrane-anchored serine/threonine-specific protein kinases, is related to the coordination of critical physiological processes, including apoptosis

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or neurogenesis [4,8,14]. As a brain enriched neuronal kinase, LMTK2 has attracted wide attention in neurodegenerative disease research due to its regulation of numerous neurodegeneration-related fundamental cellular pathways [4]. Previous studies have demonstrated that LMTK2 was downregulated in the brains of patients with AD and AD mouse models, highlighting the potential therapeutic target of LMTK2 for AD treatment, and also disclosed three important cellular mechanisms of LMTK2 in AD, including axonal transport, tau hyperphosphorylation and apoptosis [4,6,17].

Nuclear factor erythroid 2-related factor (Nrf2) is crucial in regulating the redox equilibrium. The translocation of activated Nrf2 into the nucleus triggers the transcription of antioxidative enzyme through interacting with the antioxidant responsive element (ARE), an enhancer element that regulates detoxification genes [15,20]. Currently, the dysfunction of the Nrf2-ARE signalling-related pathway has been widely evidenced to a critical contributor to neurological pathologies, including AD, and activation of Nrf2-ARE was demonstrated to exert a neuroprotective role [12,25]. Therefore, an in-depth investigation into the neuroprotective properties and potential mechanism of the involvement of the Nrf2-ARE pathway in the aetiology of neurodegenerative diseases is indispensable to develop novel therapeutic modalities to suppress the advancement of many neurological diseases [11].

β-amyloid (Aβ) is a vital pathophysiological feature of AD, and Aβ-stimulated apoptosis and necrosis of neurons have been shown to result in the production of reactive oxygen species (ROS) in cells [24]. What is worse, cerebral Aβ accumulation could cause neurotoxicity and dysfunction of neurons via modulation of the structure and plasticity of synapses in AD patients [23]. Thus, we adopted $A\beta_{25-35}$ to induce PC12 cells to construct an AD cell model *in vitro*. The work was designed to explore the impacts of LMTK2 on AD and to identify the hidden regulatory mechanism.

Material and methods

Cell culture

PC12 cells (Yaji Biotechnology Co., Ltd., Shanghai, China) were inoculated into DMEM which contains 15% FBS (Sijiqing Biologic Co. Ltd., Zhejiang, China) and 1% antibiotics at 37°C with 5% CO_2.

Grouping and plasmid transfection

Cells were classified into Control (untreated cells), $A\beta_{25-35}$ (cells incubated with 20 μmol/l $A\beta_{25-35}$ for 24 h), $A\beta_{25-35}$ + oe-NC (the cells were transfected with overexpression negative control (oe-NC) prior to the incubation with 20 μmol/l $A\beta_{25-35}$) as well as $A\beta_{25-35}$ + oe-LMTK2 groups (the cells were transfected with LMTK2 overexpression plasmid (oe-LMTK2) prior to the incubation with 20 μmol/l $A\beta_{25-35}$). In addition, for further research on Nrf2-ARE signalling, PC12 cells were treated with ML385 (5 μM, 1 h pre-treatment), a specific inhibitor of Nrf2.

With the employment of Lipofectamine 2000 provided by Invitrogen Life Technologies (Shanghai, China), 1 μg oe-LMTK2 or oe-NC plasmids (Nanjing KeyGEN BioTECH) were transfected into PC12 cells inoculated in six-well plates (2×10^5 cells/well).

Reverse transcription-quantitative PCR (RT-qPCR)

With the help of RT Easy™ I (cat. no. RT-01011; ForeGene), the RNA isolated by Cell Total RNA Isolation Kit (cat. no. RE-03111; ForeGene) was reverse transcribed into complementary DNA (cDNA) in line with the manufacturer's guidelines. By using the ABI 7500 quantitative PCR instrument (cat. no. A24820; Applied Biosystems), PCR amplification was conducted *via* SYBR Green I (cat. no. QP-01012; ForeGene). Finally, the $2^{-\Delta\Delta Ct}$ method was used for the calculation of alternations in gene expression, followed by normalization against GAPDH.

Western blot

The extraction and quantification of proteins from each group of PC12 cell were conducted with RIPA lysis buffer (cat. no. AS1004; ASPEN, Wuhan, China) and bicinchoninic acid (BCA) protein assay kit (cat. no. AS1086; ASPEN, Wuhan, China), respectively. 10% SDS-PAGE gels-separated protein samples (20 μg) were electroblotted onto a PVDF membrane. After incubation with 5% skimmed milk for 2 h. The membranes were probed with primary antibodies at 4°C overnight, followed by HRP-conjugated goat anti-rabbit secondary antibody at room temperature for 2 h. Finally, an enhanced chemiluminescence reagent (ASPEN, Wuhan, China) was adopted for the development of the protein bands.

Cell Counting Kit 8

The supplementation of 10 μl Cell Counting Kit 8 (CCK-8) reagent (Beijing Transgen Biotech, China) to PC12 cells inoculated into 96-well plates was conducted, followed by incubation for 2 h at 37°C. Under a microplate reader, the absorbance at a wavelength of 450 nm was appraised.

Detection of oxidation stress

A total of 200 μl supernatant was harvested after the collected culture medium was centrifugated at

10,000 × g for 10 min at 4°C. Subsequently, malondialdehyde (MDA; cat. no. S0131M), superoxide dismutase (SOD; cat. no. S0086) and catalase (CAT; cat. no. S0082) contents were separately evaluated with related assay kits from Beyotime Biotechnology (Shanghai, China) in line with the manufacturer's guidelines.

Terminal-deoxynucleotidyl Transferase Mediated Nick End Labelling (TUNEL)

Cell apoptosis were assessed with a TUNEL kit (Servicebio, China). In brief, 15 min of immobilization and 20 min of permeabilization were separately performed using 4% paraformaldehyde and 0.25% Triton X-100 at room temperature. Then, labelling reaction and nuclear staining were respectively conducted utilizing TUNEL reagent for 1 h and 1 µg/ml DAPI in the dark. Eventually, under a florescent microscope (magnification, 200×; Olympus Corporation), TUNEL-positive apoptotic cells were observed.

Measurement of Fe^{2+} and reactive oxygen species

For iron detection, the supernatant of culture medium was collected. With the application of iron colorimetric assay kit (cat. no. K390; Biovision), the iron concentration was assessed. To measure the content of ROS, PC12 cells were incubated with 10 µmol/l DCFH-DA (Molecular Probes, China). Thereafter, a fluorescence microplate reader was employed to observe the fluorescence at the excitation/emission 488/525 nm following PBS washing.

Statistical analyses

Experimental data analyzed by Prism 8.0 software (GraphPad, La Jolla, CA) are given in the format of mean value ± standard deviation (SD). One-way analysis of variance (ANOVA) as well as Turkey's test were utilized to compare differences of multiple groups. $P < 0.05$ was the threshold of statistical significance.

Results

The expression of LMTK2 in Aβ$_{25-35}$-induced PC12 cells

To confirm the reliability of the following experimental data, LMTK2 expression was firstly tested. As Figure 1A, B demonstrated, relative to the Control group, LMTK2 mRNA and protein expression in PC12 cells were overtly decreased following Aβ$_{25-35}$ induction. Further, the decreased LMTK2 expression in PC12 cells challenged with Aβ$_{25-35}$ was then upregulated by transfection of oe-LMTK2 plasmid. In addition,

the diminished cell viability due to Aβ$_{25-35}$ induction in PC12 cells was subsequently elevated after LMTK2 was overexpressed, revealing the protective role of LMTK2 elevation against Aβ$_{25-35}$-stimulated PC12 cell viability loss (Fig. 1C).

LMTK2 overexpression inhibits the oxidative stress in Aβ$_{25-35}$-induced PC12 cells

In order to explore the effects of LMTK2 overexpression on the oxidative stress in PC12 cells exposed to Aβ$_{25-35}$, MDA, SOD and CAT contents were examined with related assay kits. Aβ$_{25-35}$ induction upregulated the MDA level but downregulated SOD and CAT levels relative to the Control group. However, the impacts of Aβ$_{25-35}$ exposure on MDA, SOD and CAT were abolished by LMTK2 elevation, evidenced by the depleted MDA level and raised SOD and CAT levels in the Aβ$_{25-35}$ + oe-LMTK2 group (Fig. 1D-F). Moreover, the expressions of oxidative stress-related proteins including NOX2 and NOX4 were measured by western blot. Results in Figure 1G revealed that Aβ$_{25-35}$ induction markedly upregulated the expressions of NOX2 and NOX4 while LMTK2 overexpression reversed that trend, testified by the downregulated NOX2 and NOX4 expressions in the Aβ$_{25-35}$ + oe-LMTK2 group.

LMTK2 overexpression inhibits the apoptosis damage in Aβ$_{25-35}$-challenged PC12 cells

The apoptosis as well as its related proteins was detected with the help of TUNEL and western blot. Compared with the Control group, the apoptosis level was greatly increased in Aβ$_{25-35}$-induced PC12 cells, which was then diminished after overexpressing LMTK2 expression (Fig. 2A). Besides, Aβ$_{25-35}$ induction decreased Bcl-2 expression whereas increased Bax, cleaved caspase-3 and cleaved caspase-9 expressions by contrast with that in the Control group while LMTK2 expression exhibited opposite effects on them, as uncovered by the increased Bcl-2 expression and decreased Bax, cleaved caspase-3 and cleaved caspase-9 expressions in the Aβ$_{25-35}$+oe-LMTK2 group (Fig. 2B).

LMTK2 overexpression inhibits the ferroptosis in Aβ$_{25-35}$-exposed PC12 cells

To disclose whether LMTK2 overexpression participated in Aβ$_{25-35}$-triggered ferroptosis in PC12 cells, ROS and Fe^{2+} contents were evaluated with corresponding assay kits. Notably, Aβ$_{25-35}$ induction markedly improved the content of ROS in PC12 cells relative to the Control

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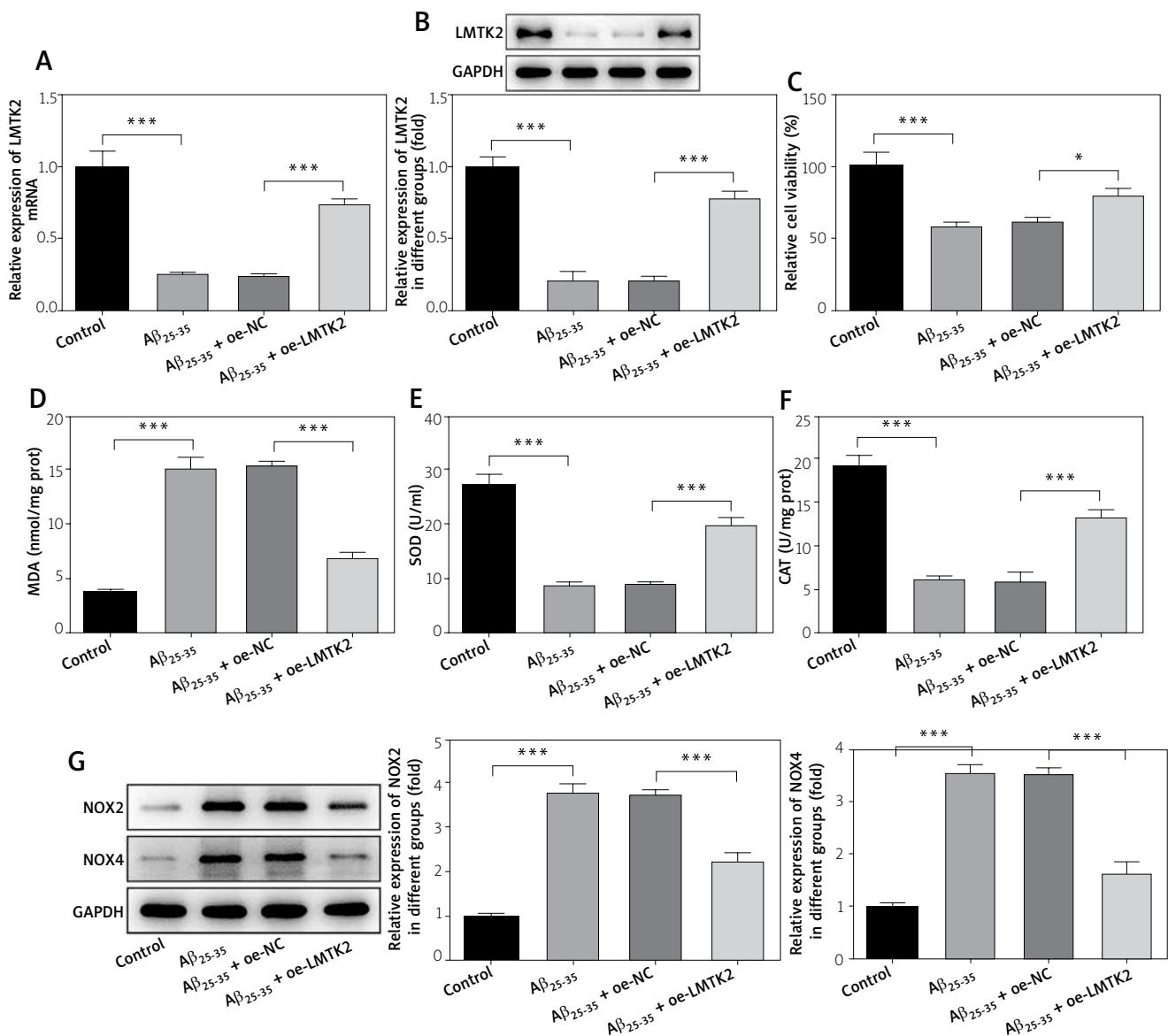
Fig. 1. LMTK2 overexpression inhibits the oxidative stress in $A\beta_{25-35}$-induced PC12 cells. **A**) The mRNA expression of LMTK2 was detected using RT-qPCR. **B**) The protein expression of LMTK2 was detected using western blot. **C**) The cell viability was detected using CCK-8. **D-F**) The levels of MDA, SOD and CAT were measured using corresponding assay kits. **G**) The expressions of NOX2 and NOX4 were detected using western blot. *$p < 0.05$, ***$p < 0.001$.

group while LMTK2 overexpression partially abrogated the elevated ROS production on account of $A\beta_{25-35}$ induction, evidenced by the decreased ROS content in the $A\beta_{25-35}$ + oe-LMTK2 group (Fig. 3A). Likewise, the increased Fe^{2+} level in $A\beta_{25-35}$-induced PC12 cells was subsequently decreased after transfection with LMTK2 overexpression plasmids (Fig. 3B). What is more, $A\beta_{25-35}$ induction enhanced ACSL4 expression while reduced GPX4 and FTH1 expressions in PC12 cells relative to the Control group (Fig. 3C). However, ACSL4 expression was

depleted and GPX4 and FTH1 expressions were augmented in PC12 cells in the $A\beta_{25-35}$ + oe-LMTK2 group.

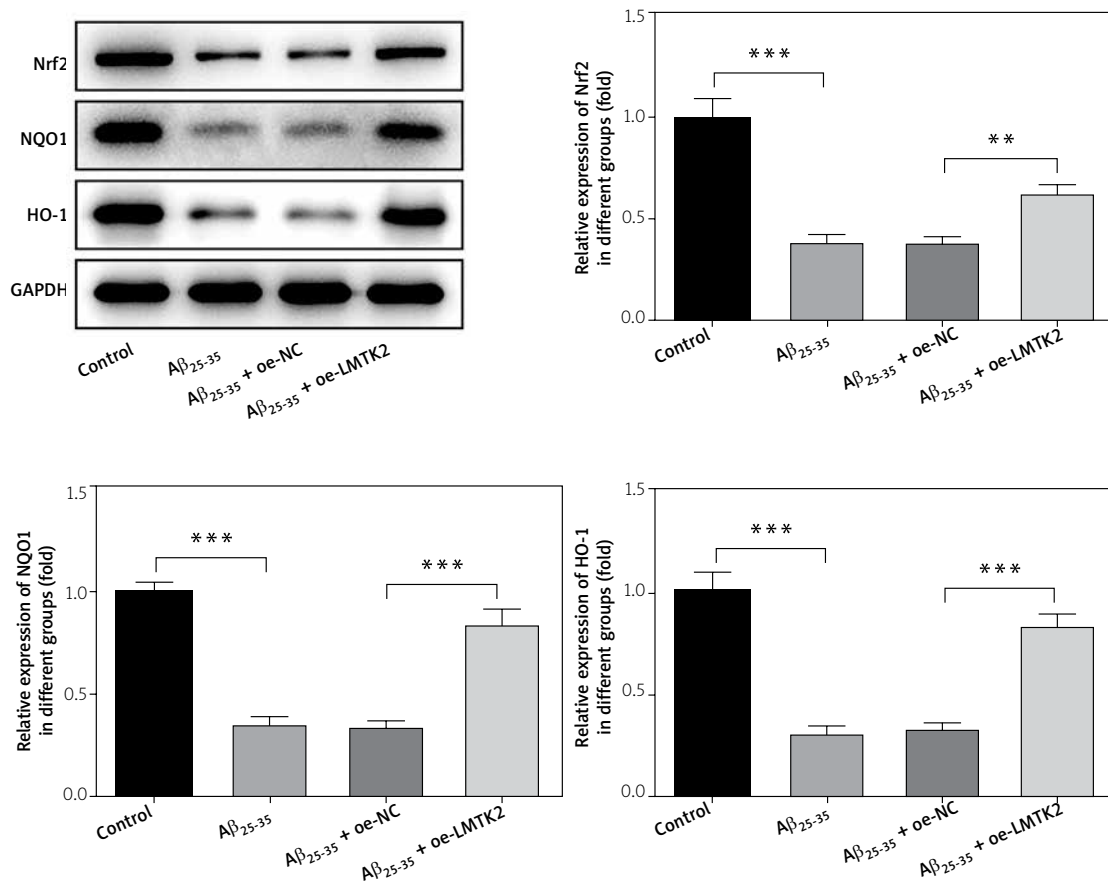## LMTK2 overexpression activates the Nrf2/ARE signalling pathway

As Figure 4 depicted, Nrf2, NQO1 and HO-1 expressions were greatly diminished in PC12 cells after $A\beta_{25-35}$ induction relative to that in the Control group. Besides, by contrast with $A\beta_{25-35}$ + oe-NC, LMTK2 overexpression raised Nrf2, NQO1 and HO-1 expressions, reveal-
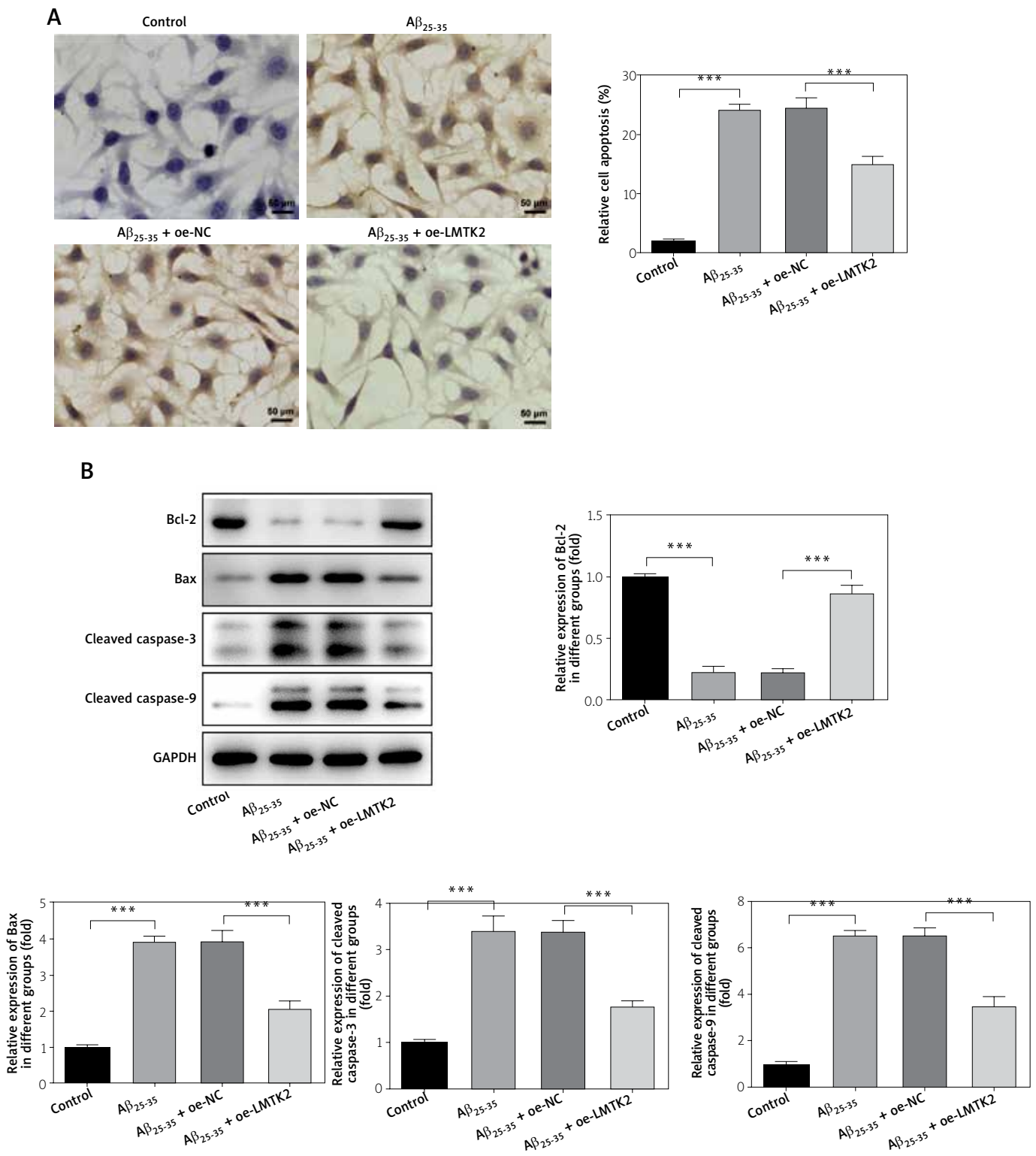
Fig. 2. LMTK2 overexpression inhibits the apoptosis damage in Aβ$_{25-35}$-induced PC12 cells. **A**) The apoptosis was detected using TUNEL. **B**) The expressions of apoptosis-related proteins were detected using western blot. ***$p < 0.001$.

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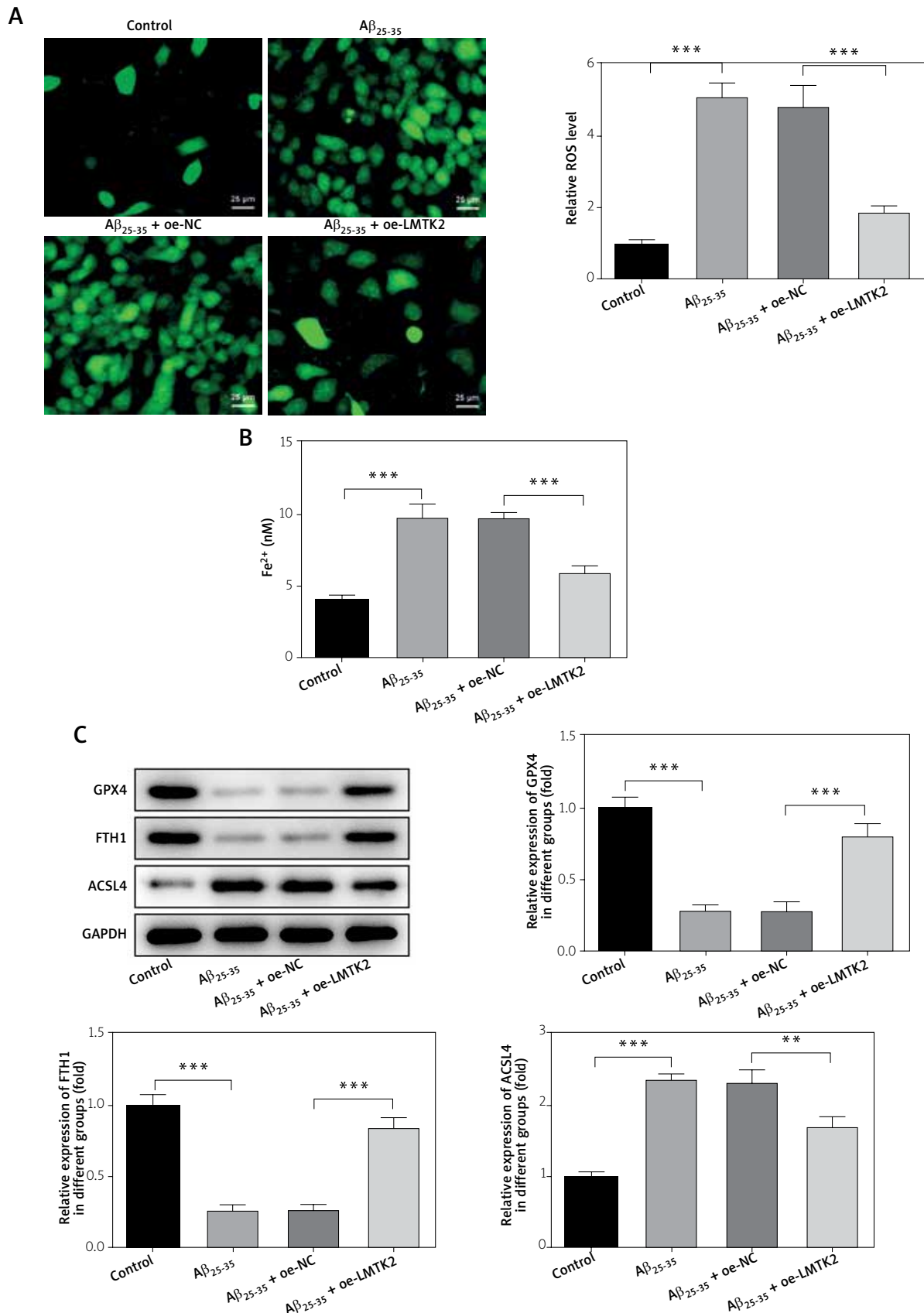
Fig. 3. LMTK2 overexpression inhibits the ferroptosis in $A\beta_{25-35}$-induced PC12 cells. **A**) The ROS content was detected using DCFH-DA. **B**) The level of Fe^{2+} was detected using the iron colorimetric assay kit. **C**) The expressions of ferroptosis-related proteins were detected using western blot. $**p < 0.01$, $***p < 0.001$.

Fig. 4. LMTK2 overexpression activates the Nrf2/ARE signalling pathway. The expressions of the Nrf2/ARE signalling pathway-related proteins were detected using western blot. $**p < 0.01$, $***p < 0.001$.
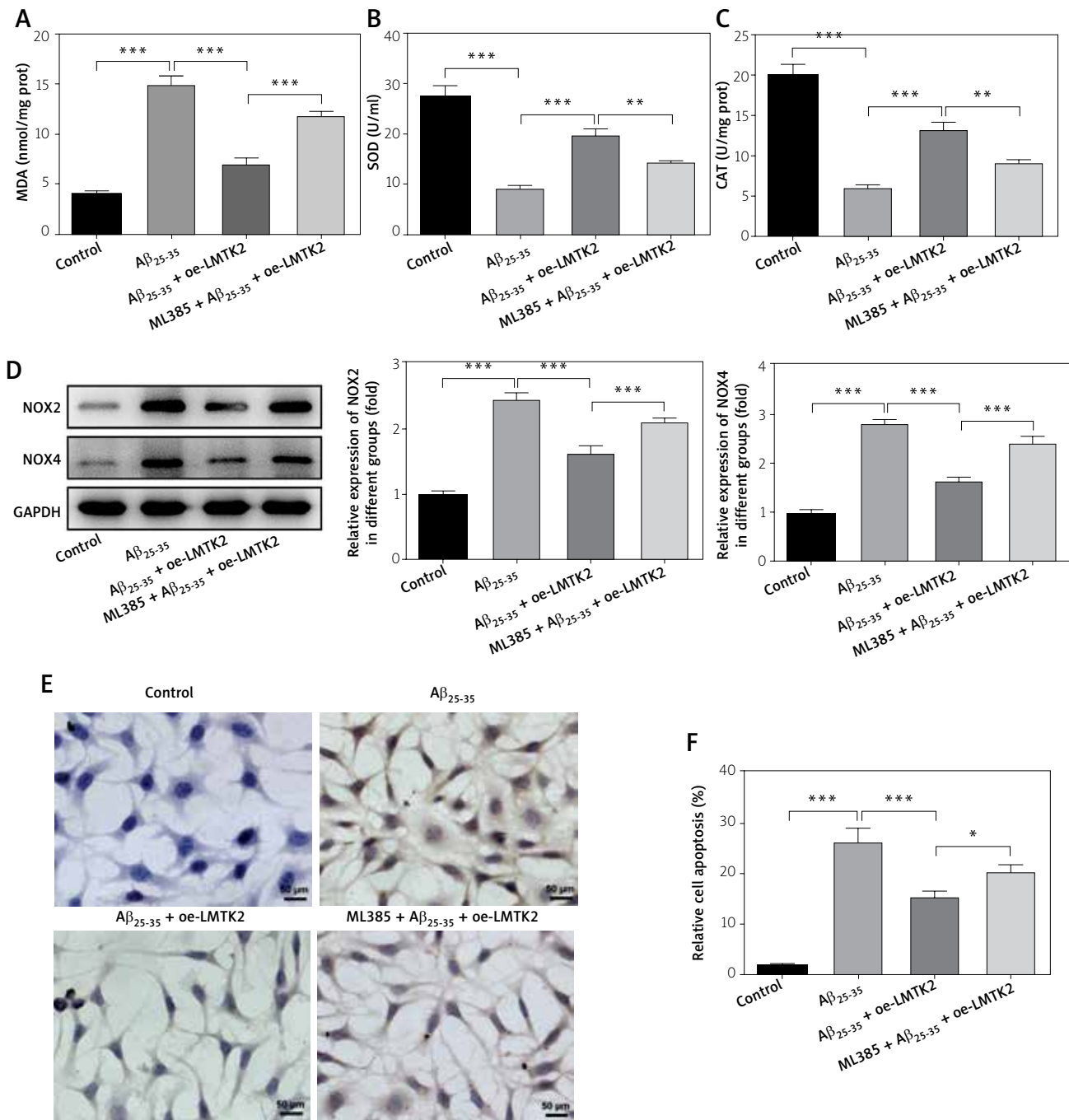
ing that LMTK2 overexpression could activate the Nrf2/ARE signalling pathway.

LMTK2 overexpression inhibits the oxidative stress, apoptosis and ferroptosis in Aβ₂₅₋₃₅-induced PC12 cells *via* activating Nrf2/ARE signalling

Nrf2 inhibitor ML385 with a concentration of 5 μM was utilized to treat PC12 cells to further figure out the mechanism of LMTK2 elevation. Evidently, LMTK2 overexpression downregulated MDA expression but upregulated SOD and CAT expressions in Aβ₂₅₋₃₅-exposed PC12 cells. The addition of ML385 partially fortified MDA expression and cut down SOD and CAT expressions in the ML385 + Aβ₂₅₋₃₅ + oe-LMTK2 group (Fig. 5A-C). Additionally, ML385 greatly enhanced the levels of NOX2 and NOX4 relative to that in the Aβ₂₅₋₃₅ + oe-LMTK2 group (Fig. 5D). The apoptosis was reduced after transfection with oe-LMTK2 plasmid by contrast with the Aβ₂₅₋₃₅

group. However, ML385 administration increased the apoptosis in contrast with that in the Aβ₂₅₋₃₅ + oe-LMTK2 group (Fig. 5E, F). Moreover, the ascending Bcl-2 expression but descending Bax, cleaved-caspase3 and cleaved-caspase9 expressions in the Aβ₂₅₋₃₅ + oe-LMTK2 group were reversed after ML385 administration, suggesting that LMTK2 overexpression obstructed Aβ₂₅₋₃₅-elicited PC12 cell apoptosis *via* activating the Nrf2/ARE signalling pathway (Fig. 5G).

Moreover, the decreased ROS level in Aβ₂₅₋₃₅ + oe-LMTK2 was enhanced by ML385 administration (Fig. 5H). Similarly, LMTK2 overexpression reduced the level of Fe^{2+} in comparison with that in the Aβ₂₅₋₃₅ group while ML385 raised Fe^{2+} level in the ML385 + Aβ₂₅₋₃₅ + oe-LMTK2 group (Fig. 5I). What is more, ML385 treatment enhanced ACSL4 expression whilst diminished GPX4 and FTH1 expressions relative to the Aβ₂₅₋₃₅ + oe-LMTK2 group, implying that LMTK2 overexpression inhibited the ferroptosis in Aβ₂₅₋₃₅-treated PC12 cells *via* activating the Nrf2/ARE signalling pathway.

Fig. 5. MTK2 overexpression inhibits the oxidative stress, apoptosis damage and ferroptosis in $A\beta_{25-35}$-induced PC12 cells *via* activating the Nrf2/ARE signalling pathway. **A-C)** The levels of MDA, SOD and CAT were detected using corresponding assay kits. **D)** The expressions of NOX2 and NOX4 were detected using western blot. **E, F)** The apoptosis was detected using TUNEL.
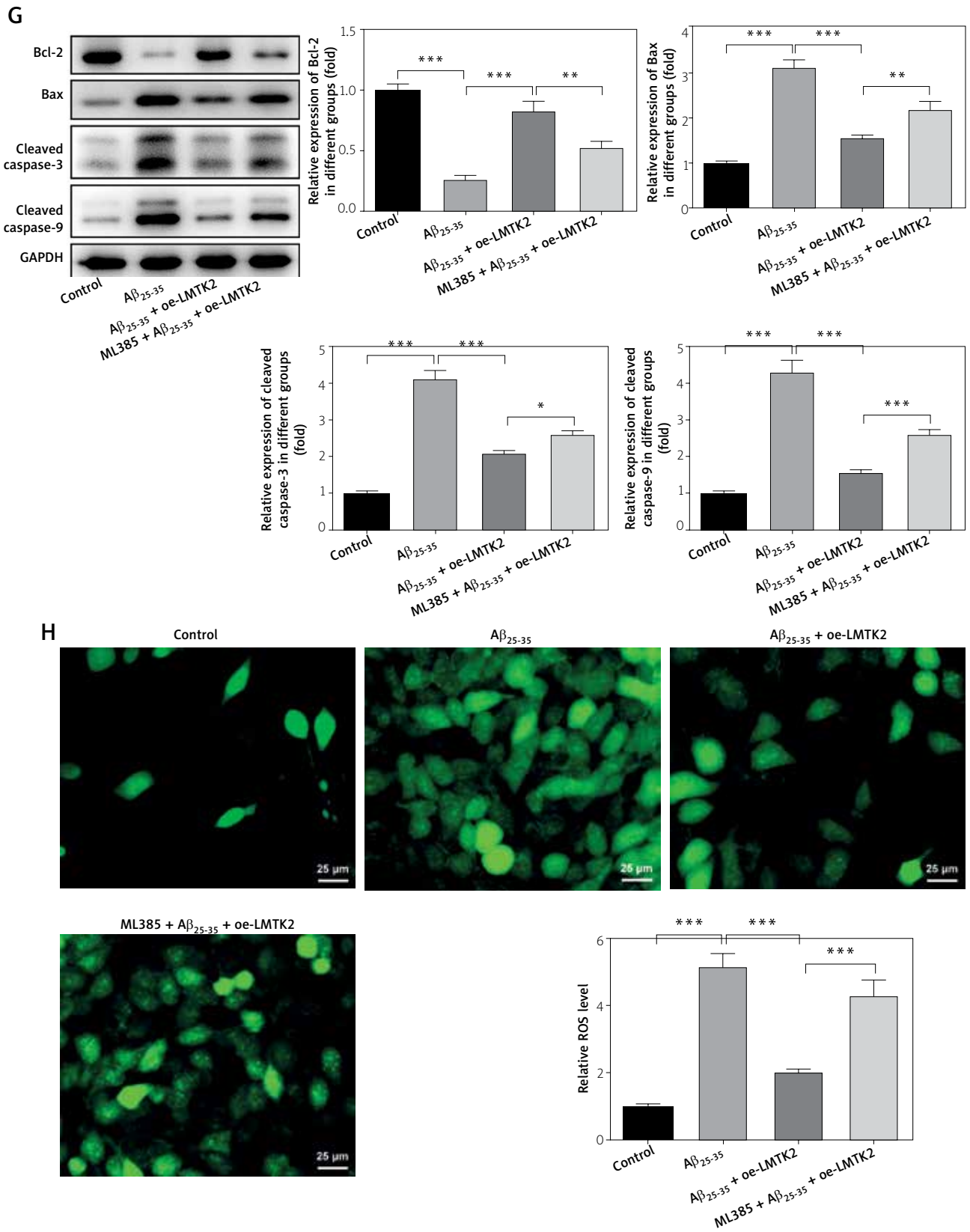
Fig. 5. Cont. **G**) The expressions of apoptosis-related proteins were detected using western blot. **H**) The ROS content was detected using DCFH-DA.
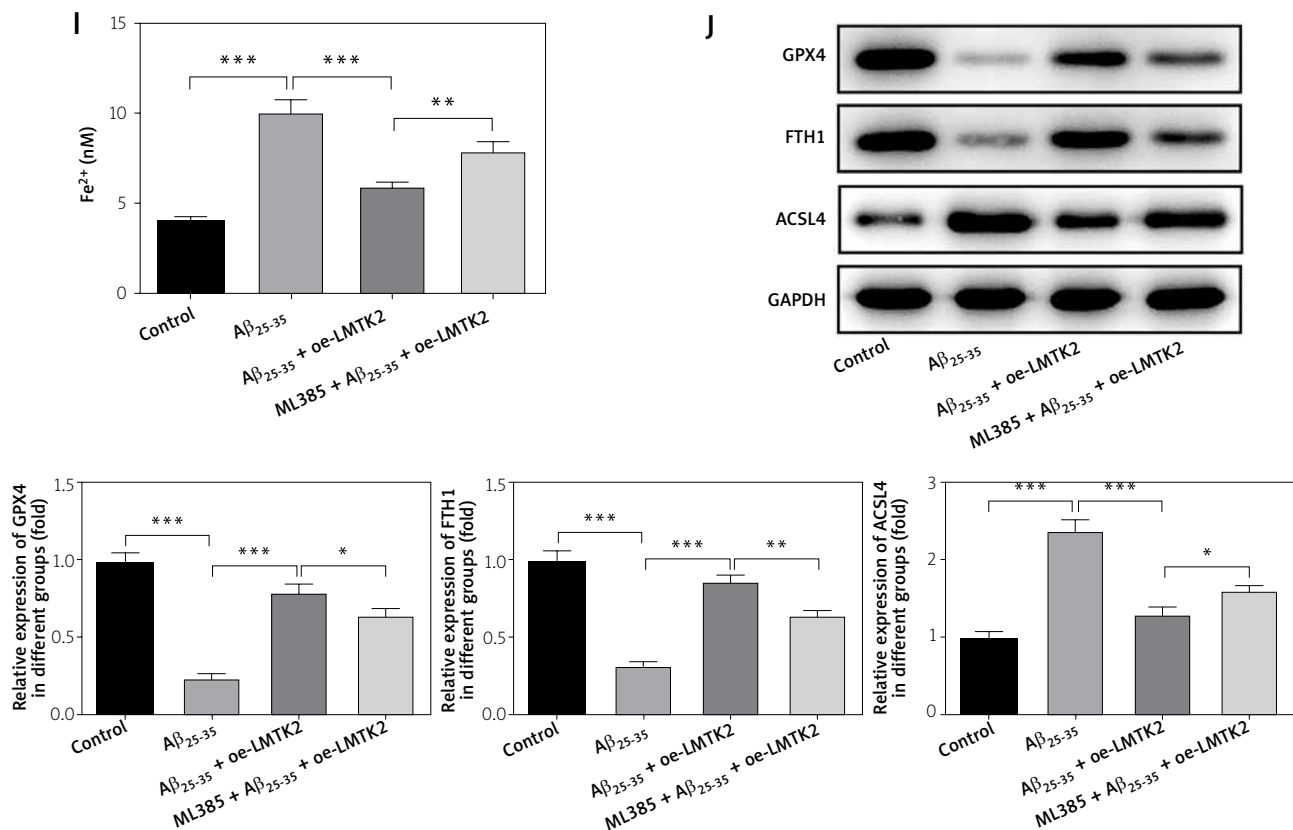
Fig. 5. Cont. **I)** The level of Fe^{2+} was detected using the iron colorimetric assay kit. **J)** The expressions of ferroptosis-related proteins were detected using western blot. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Discussion

To our knowledge, we firstly explored the influence of LMTK2 in PC12 cells challenged with $A\beta_{25-35}$ and its relation with the Nrf2/ARE signalling pathway. Initially, it was noted that LMTK2 expression was markedly declined in $A\beta_{25-35}$-exposed PC12 cells. Then, the gain-of-function experiments and a series of cellular biological experiments indicated that LMTK2 overexpression inhibited the oxidative stress, apoptosis and ferroptosis of $A\beta_{25-35}$-challenged PC12 cells but promoted the cell viability. Besides, it was also testified that LMTK2 overexpression activated the Nrf2/ARE signalling pathway. What is more, the mitigated oxidative stress, apoptosis and ferroptosis in LMTK2-overexpressing PC12 cells exposed to $A\beta_{25-35}$ was partly potentiated by ML385, highlighting that LMTK2 might exert a protective role via activating the Nrf2/ARE signalling pathway.

Alzheimer's disease, which was firstly identified over 100 years ago, was considered to be the leading cause of human death [13]. Oxidative stress, an important pathophysiological change in AD, promotes $A\beta$ deposition, tau hyperphosphorylation as well as the loss of synapses and neurons to be involved in

the advancement of AD [7]. Besides, apoptosis could induce the occurrence of deleterious neurodegenerative diseases when it was impaired or affected by factors like Bcl2 and Bax [18]. Moreover, ferroptosis was also a vital player in neuronal death and neurological disorders, meanwhile iron depositions could be detected in the specific brain regions in AD [1]. Considering above, we can speculate that the underlying mechanisms of AD may involve oxidative stress, apoptosis and ferroptosis. As a neuronal kinase abundant in the brain, LMTK2 was testified to be downregulated in AD [17]. Additionally, it was reported that LMTK2 overexpression could attenuate neuronal apoptosis and oxidative damage [3]. In our study, in compliance with the previous study, we found that LMTK2 was decreased in $A\beta_{25-35}$-induced PC12 cells [17]. It was also discovered that MDA, NOX2, NOX4, ROS and Fe^{2+} activities were downregulated in PC12 cells challenged with $A\beta_{25-35}$ after transfection of oe-LMTK2 plasmid, revealing the protective role of LMTK2 overexpression against oxidative stress and ferroptosis in AD. What is more, LMTK2 overexpression was testified to inhibit apoptosis in $A\beta_{25-35}$-induced PC12 cells through lessening pro-apop-

totic Bax expression, which indicated that LMTK2 overexpression imparted suppressive effects on apoptosis in AD.

The Nrf2/ARE signalling pathway acts as a critical player in defending against oxidative stress, apoptosis, inflammation as well as neuron damage in response to oxidative stimuli [16]. A previous study testified that Nrf2 mediated neuroprotection by upregulating NQO1, HO-1, and superoxide dismutase 2 (SOD2) expressions [10]. In addition, the overexpression of LMTK2 induced elevated Nrf2, as well as HO-1 and NQO1 expressions [19]. Furthermore, it was demonstrated that LMTK2 overexpression could strengthen Nrf2/ARE antioxidant signalling by regulating GSK-3β phosphorylation [3]. In our experiments, we discovered that Nrf2, NQO1, and HO-1 were significantly decreased in Aβ$_{25-35}$-induced PC12 cells, which were then increased following LMTK2 elevation, suggesting that LMTK2 overexpression could activate the Nrf2/ARE pathway.

Then the effects of the Nrf2/ARE signalling pathway on LMTK2 overexpression-inhibited apoptosis, oxidative stress and ferroptosis in PC12 cells exposed to Aβ$_{25-35}$ through ML385, a Nrf2 inactivator, were tracked to further figure out the specific molecular mechanisms of Nrf2/ARE signalling. In this study, it was noted that the reduced oxidative stress caused by LMTK2 overexpression in Aβ$_{25-35}$-induced P12 cells was increased by ML385 administration, indicating that the block of the Nrf2/ARE signalling pathway promoted the oxidative stress in AD. What is more, the weakened apoptosis and ferroptosis of Aβ$_{25-35}$-induced P12 cells following LMTK2 overexpression were observed to be strengthened after ML385 treatment. These findings implied that LMTK2 overexpression exhibited inhibitory effects on the Aβ$_{25-35}$-triggered oxidative stress, apoptosis and ferroptosis in P12 cells *via* activating the Nrf2/ARE signalling pathway.

Taken together, the association of LMTK2 with Nrf2/ARE signalling was initially introduced in AD, highlighting the protective effects of LMTK2 on AD. Based on the results of our study, more effects need to be made to examine LMTK2 as a potential therapeutic target in AD via animal studies and clinical investigation.

Disclosure

The authors report no conflict of interest.

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