CB1 receptor mediates anesthetic drug ketamine-induced neuroprotection against glutamate in HT22 cells

HE BAO[1], CHEN WANG[2], XIAORONG XUE[3], BIN HU[3] and QI GUO[3]

[1]Department of Pharmacy, The Second Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi 710004;
[2]Department of Pharmacy, The Second Affiliated Hospital of Air Force Medical University, Xi'an, Shaanxi 710038;
[3]Department of Pharmacy, Xi'an People's Hospital (Xi'an Fourth Hospital), Xi'an, Shaanxi 710004, P.R. China

Abstract. The anesthetic drug, ketamine (KTM) has been shown to induce therapeutic effects against major depressive disorder (MDD), however the related underlying mechanisms remain unclear. In the present study, HT22 neuronal cells were treated with glutamate to imitate oxidative stress injury in MDD, and it was hypothesized that the cannabinoid type 1 (CB1) receptor mediates KTM-induced neuroprotection via ameliorating mitochondrial function in glutamate-treated neuronal cells. Compared with the control, glutamate decreased cell viability and intracellular antioxidants, including glutathione (GSH), catalase and superoxide dismutase 2 levels, and inhibited mitochondrial function simultaneously. Moreover, glutamate increased lactate dehydrogenase release, cellular apoptosis level, cleaved caspase-3 expression and intracellular oxidants, such as reactive oxygen species, oxidized GSH and mitochondrial superoxide in the cells. The presence of KTM, however, significantly decreased the glutamate-induced oxidative stress injury, ameliorated the antioxidant/oxidant levels in the cells, enhanced mitochondrial function and upregulated CB1 receptor expression (P<0.05). Co-administration of the CB1 receptor antagonist AM251 markedly abolished the KTM-induced cytoprotective effects and ameliorations of antioxidant/oxidant levels and mitochondrial function, and also reversed CB1 upregulation (P<0.05). These observations indicated that KTM decreases the oxidative stress injury caused by glutamate in HT22 neuronal cells, and the neuroprotective effects may be mediated by the CB1 receptor.

Correspondence to: Professor He Bao, Department of Pharmacy, The Second Affiliated Hospital of Xi'an Jiaotong University, 157 Xiwu Road, Xi'an, Shaanxi 710004, P.R. China
E-mail: baohe2yuan@xjtu.edu.cn

Professor Qi Guo, Department of Pharmacy, Xi'an People's Hospital (Xi'an Fourth Hospital), 41 Jiefang Road, Xi'an, Shaanxi 710004, P.R. China
E-mail: foox55555@163.com

Introduction

Major depressive disorder (MDD) is a common psychiatric condition, and it is predicted that MDD affects ~264 million individuals globally (1,2). In China, the incidence of MDD is ~3.4%, and in the USA, the incidence is up to 16.2%, causing a heavy burden for the families of patients with MDD and the nation as a whole (3). At present, traditional anti-depressants, such as mono-amine oxidase inhibitors, tricyclic agents and selective serotonin reuptake inhibitors, require up to six weeks to induce obvious anti-depressive effects (4,5). As patients with MDD carry a high risk of death by suicide, it is urgent that solutions are found to reduce suicidal tendencies amongst these individuals (6,7). Therefore, investigations into the development of a fast-acting anti-depressants are of great importance. Previously, a number of studies have shown that ketamine (KTM), an intravenous anesthetic agent, is effective in treating MDD, and notably has been revealed to reduce suicidal tendencies in patients with MDD in a short period of time (several days to 1-2 weeks) (6,8). Although KTM is effective in treating MDD, the anti-depressive mechanism of KTM remains unknown. Traditionally, it has been hypothesized that KTM induces pharmacological effects via inhibition of the cellular N-methyl-D-aspartic acid receptor. However, recent studies have demonstrated that cannabinoid (CB) receptors, including CB1 and CB2 receptors, are involved in KTM-induced anti-depressive effects (9,10).

In the pathological processes of MDD, oxidative injury-induced mitochondrial dysfunction plays a critical role (11,12). In cells, mitochondria produce energy for cellular activities, however in the leukocytes of patients with MDD, a lower mitochondrial DNA copy number was recorded compared with individuals without MDD (13,14). In addition, serum antioxidant levels in patients with MDD are notably lower than that of healthy individuals (15). Moreover, the cerebral glutamate level in patients with MDD is higher than healthy individuals (15). Due to the aforementioned reasons, glutamate-induced cell injury is used widely to mimic oxidative stress injury in neurons (16).

Therefore, in the present study, HT22 neuronal cells were treated with glutamate to imitate oxidative injury in MDD, and it was hypothesized that the CB1 receptor mediates KTM-induced protection against glutamate via ameliorating mitochondrial function in HT22 cells.

Materials and methods

Cells and reagents. HT22 cells, a murine hippocampal cell line, were obtained from The Affiliated Hospital of The Xuzhou Medical University (Jiangsu, China). KTM was purchased from Fujian Gutian Pharmaceutical Co., Ltd. (Fujian, China). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin mixed solution and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) powder were purchased from MilliporeSigma. Lactate dehydrogenase (LDH, cat. no. A020-1-2), glutathione (GSH, cat. no. A006-1-1), catalase (CAT, cat. no. A007-1-1) and oxidized GSH (GSSG, cat. no. A061-2-1) reagent kits were obtained from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China).

Cell culture and treatments. HT22 cells were cultured in DMEM containing 10% FBS (v:v) and 1% penicillin-streptomycin mixed solution ($1x10^4$ IU penicillin and $1x10^4$ μg streptomycin/ml). The medium was changed 3 times/week, and the cells were cultured at 37˚C in 95% oxygen and 5% carbon dioxide with 100% humidity. The cells were passaged 2-3 times/week.

The cells were divided into five groups to determine an ideal concentration of KTM. The groups were as follows: i) Control group (cultured in the medium without any drug); ii) glutamate exposure group (exposed to the medium with 15 mM glutamate); iii) 1 μg/ml KTM treatment group (exposed to the medium with 1 μg/ml KTM plus 15 mM glutamate); iv) 10 μg/ml KTM treatment group (exposed to the medium with 10 μg/ml KTM plus 15 mM glutamate); and v) 20 μg/ml KTM group (exposed to the medium with 20 μg/ml KTM plus 15 mM glutamate). After incubation for 24 h, the degree of cell injury was assessed by determining the cell viability and LDH release.

Next, the role of the CB1 receptor in the KTM-treated cells was investigated and the cells were divided into five groups, including: i) Control group; ii) 15 mM glutamate group; iii) 10 μg/ml KTM + 15 mM glutamate group; iv) 10 μM CB1 antagonist AM251 + 10 μg/ml KTM + 15 mM glutamate group; and v) 10 μM AM251 + 15 mM glutamate group. After incubation for 24 h, the degree of cell injury and intracellular oxidant/antioxidant levels were assessed. Moreover, to explore the KTM-induced effects on the intracellular superoxide dismutase (SOD) 1 and SOD2 levels, the cells were divided into the control and 10 μg/ml KTM exposure groups. After treatment for 24 h, intracellular SOD1/SOD2 activity and expression levels were measured.

MTT and LDH assay. A 96-well cell culture plate was used to culture the cells with a cell intensity of $1x10^5$ cells/well. As the treatments were completed, 20 μl of MTT solution with a concentration of 5 mg/ml was added to each plate well, and the plate was sent back into the incubator. After incubation for 4 h at 37˚C, the medium was removed and discarded, and the generated formazan of the plate was dissolved by adding 150 μl DMSO into each well. After shaking for 15 min using an agitator, once the formazan was dissolved completely, an enzyme-labeled instrument (Tecan Group, Ltd.) was used to measure the absorbance. An empty well containing 150 μl distilled water was added to serve as a blank control.

A 96-well cell culture plate was used to culture the cells and upon treatment completion, 50 μl of supernatant of the cell culture medium was collected and centrifuged at a speed of 400 x g for 30 min at room temperature. After centrifugation for 30 min, as previously described (17), 40 μl of the medium from each well was harvested to measure the LDH release level.

Cell apoptosis. A 6-well plate was used to culture the cells with a cell density of $1x10^6$ cells/well. Upon treatment completion, the medium of the plate was removed and discarded, and the cells were collected through centrifugation at a speed of 400 x g for 30 min at room temperature. A total of 2 ml phosphate-buffered saline (PBS) at 4˚C was used to wash the cells twice, and the cells were resuspended in binding buffer. Next, the anti-annexin-V staining antibody (1:1,000; cat. no. SAB5702648; MilliporeSigma), which was labelled with fluorescein isothiocyanate isomer plus propidium iodide solution, was added into the buffer. After which, the cells were mixed with the buffer slowly. In darkness at room temperature, the cells were incubated for 15 min. Finally, cell apoptosis was measured by flow cytometry (FACSCanto II; BD Biosciences), which was operated via BD FACSCanto System II Software v2.4 (BD Biosciences).

Western blot analysis. A 6-well plate was used to culture the cells with $1x10^6$ cells seeded into each well. Following treatment completion, the cells were washed with PBS three times for 5 min. After which, the cells were harvested using a cell scraper and the protein level of the cells was measured using the Bradford method. The western blotting procedure was conducted as previously described (18). The subsequent antibodies were used including anti-cleaved caspase-3 antibody (cat. no. ab231289; Abcam), anti-CB1 primary antibody (cat. no. ab259323, Abcam) and anti-SOD1/SOD2 antibody (cat. no. ab13498/ab12533; Abcam) at a dilution of 1:200. β-tubulin (1:500; cat. no. ab179511; Abcam) and β-actin (1:500; cat. no. ab8226; Abcam) served as the loading control. Image Lab Software 6.0.1 (Bio-Rad Laboratories, Inc.) was taken to analyze the images from the western blotting results.

Immunocytochemistry. A confocal microscope-specific cell culture plate was taken to culture the cells, and the cells were seeded at a density of $1x10^5$ cells/well. Upon treatment completion, the medium in the plate was discarded and PBS solution was used to wash the cells three times for 5 min. After which, the cells were incubated with 1 ml of 4% paraformaldehyde solution for 20 min, and PBS was used to wash the cells three times for 5 min. Next, the cells were incubated with 50 μl anti-CB1 primary antibody (1:50) at 4˚C for 12 h. After incubation, the primary antibody was discarded and PBS was used to wash the plate three times. Next, 100 μl Cy3-labelled goat-anti-rabbit antibody (red; 1:100; cat. no. A0516; Beyotime Institute of Biotechnology) was added into each plate; after 30-min incubation in darkness at room temperature, 50 μl DAPI solution was added into each plate. After an additional incubation in darkness for 10 min, the plate was washed again with PBS. Finally, a laser confocal fluorescence microscope (FV10i; Olympus Corporation) was used to observe the cell culture plate, and the images were captured at random.

Intracellular GSH, CAT, GSSG and SOD measurements. A 6-well plate was used to culture the cells, and $1x10^6$ cells was seeded into each well. Following cell lysis induction, 1 ml PBS (0.1 M; pH 7.4) was used. After which the cells were centrifuged at a speed of 400 x g for 20 min at 4˚C. The supernatants were harvested to measure the intracellular GSH, CAT and GSSG levels, and a spectrophotometer (Tecan Group, Ltd.) was used to detect the absorbance.

The SOD1 and SOD2 activities were measured according to an investigation described previously (19). Briefly, total SOD activity was measured first and then SOD2 activity was evaluated. As the total SOD activity is equal to the SOD1 activity plus the SOD2 activity, the SOD1 activity can be calculated according to the total SOD and SOD2 values.

Intracellular ROS assay. A confocal microscope-specific cell culture plate was used to culture the cells, and the cells were seeded at a density of $2x10^4$ cells/well. Upon treatment completion, 1 ml FBS-free cell culture medium containing 100 μM dichlorofluorescein (DCF)-DA was added into each well, after incubation for 20 min at 37˚C. After which, PBS was used to wash the cells three times for 5 min. A confocal microscope was used to observe and capture images, and the excitation and emission wavelengths were 480 and 535 nm, respectively. Non-fluorescence DCF-DA can be oxidized into fluorescence DCF (green) by intracellular ROS, with more ROS producing more DCF. Image Pro-Plus Software 6.0 (Media Cybernetics, Inc.) was used to assess the fluorescence intensity of images.

Mitochondrial complex activity assay. A 6-well plate was used to culture the cells, and $1x10^6$ cells was seeded into each well. Upon treatment completion, the cells were collected using a cell scraper. After which, the cells were treated with trypsin for 1-2 min at room temperature. Next, the cells were centrifuged at a speed of 400 x g for 30 min at room temperature for mitochondrial isolation. A mitochondrial isolation reagent kit (cat. no. 37612; Qiagen GmbH) was taken to collect and isolate the mitochondria from the cells. A spectrophotometer (Tecan Group, Ltd.) was used to measure the mitochondrial complex I and complex IV activities at 30˚C.

Mitochondrial superoxide measurement. Mitochondrial superoxide levels were evaluated using a MitoSOX Red Reagent Kit (cat. no. M36008; Invitrogen; Thermo Fisher Scientific, Inc.). Briefly, a confocal microscope-specific plate was used to culture the cells, and the cell density was $2x10^4$ cells/plate. Upon treatment completion, 5 μl MitoSOX reagent was added into each plate. In darkness, the cells were incubated for 20 min at 37˚C, and the nuclei of the cells were marked by adding 20 μl DAPI staining solution into each plate. The fluorescent images were captured at random following incubation for 15 min in darkness at room temperature. The excitation and emission wavelengths for mitochondrial superoxide (red) were 510 and 580 nm, respectively. The two wavelengths for the nuclei (blue) were 340 and 488 nm, respectively. An Image Pro-Plus Software 6.0 (Media Cybernetics, Inc.) was used to assess the fluorescence intensity of the images.

Statistical analysis. SPSS 12.0 (SPSS, Inc.) was used to conduct the statistical assessments. All data were presented as
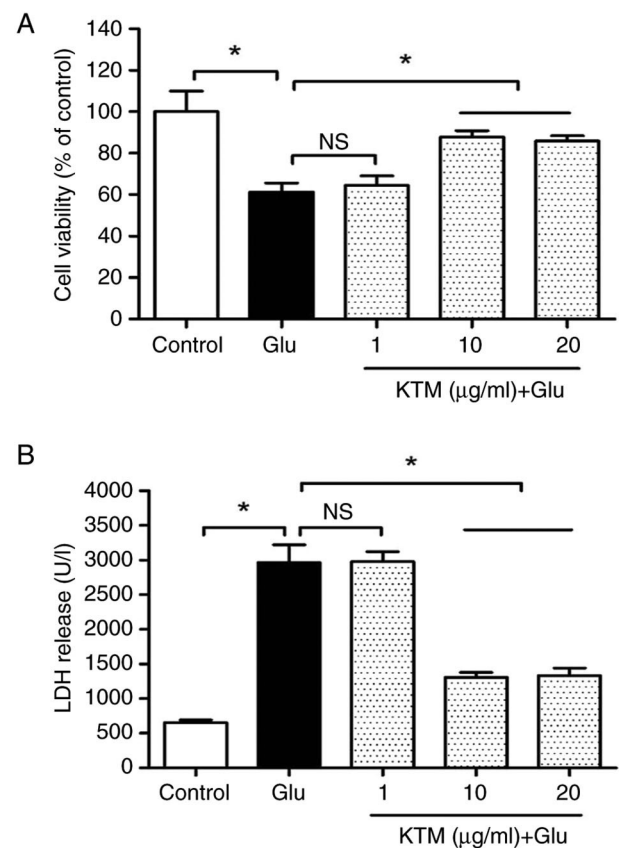

Figure 1. KTM increases cell viability and inhibits LDH release in glutamate-treated HT22 cells. The cells were grouped as follows: i) Control (cultured in drug-free medium); ii) Glu (exposed to the medium with 15 mM Glu); and iii) 3 KTM treatment groups (treated with the medium containing 1, 10 or 20 μg/ml KTM plus 15 mM Glu, respectively). MTT assay and LDH release were used to measure the degree of cell injury. (A) KTM increased the cell viability (n=10). (B) KTM decreased LDH release (n=8). *P<0.05. NS, no significance; KTM, ketamine; Glu, glutamate; LDH, lactate dehydrogenase.

the mean ± standard deviation, and one-way ANOVA followed by Tukey's Multiple Comparison Test were used to compare the differences between groups. P<0.05 was considered to indicate a statistically significant difference.

Results

KTM decreases cell injury induced by glutamate. Glutamate (15 mM) was used to imitate oxidative injury in HT22 cells. To search for a suitable KTM dose, cell viability and LDH release were used to assess the degree of cell injury (Fig. 1). Compared with the control (cultured in drug-free medium), 15 mM glutamate decreased cell viability and enhanced the release of LDH significantly (P<0.05), and 10 and 20 μg/ml KTM restored cell viability and inhibited LDH release (P<0.05). However, 1 μg/ml KTM did not induce marked protection against the cell injury caused by glutamate (P>0.05). Therefore, 10 μg/ml KTM was the selected dosage for subsequent experiments.

CB1 receptor antagonist AM251 reverses KTM-induced neuroprotection against glutamate in neuronal cells. To further explore the underlying cytoprotective mechanism of KTM, AM251, a selective CB1 receptor antagonist, was used
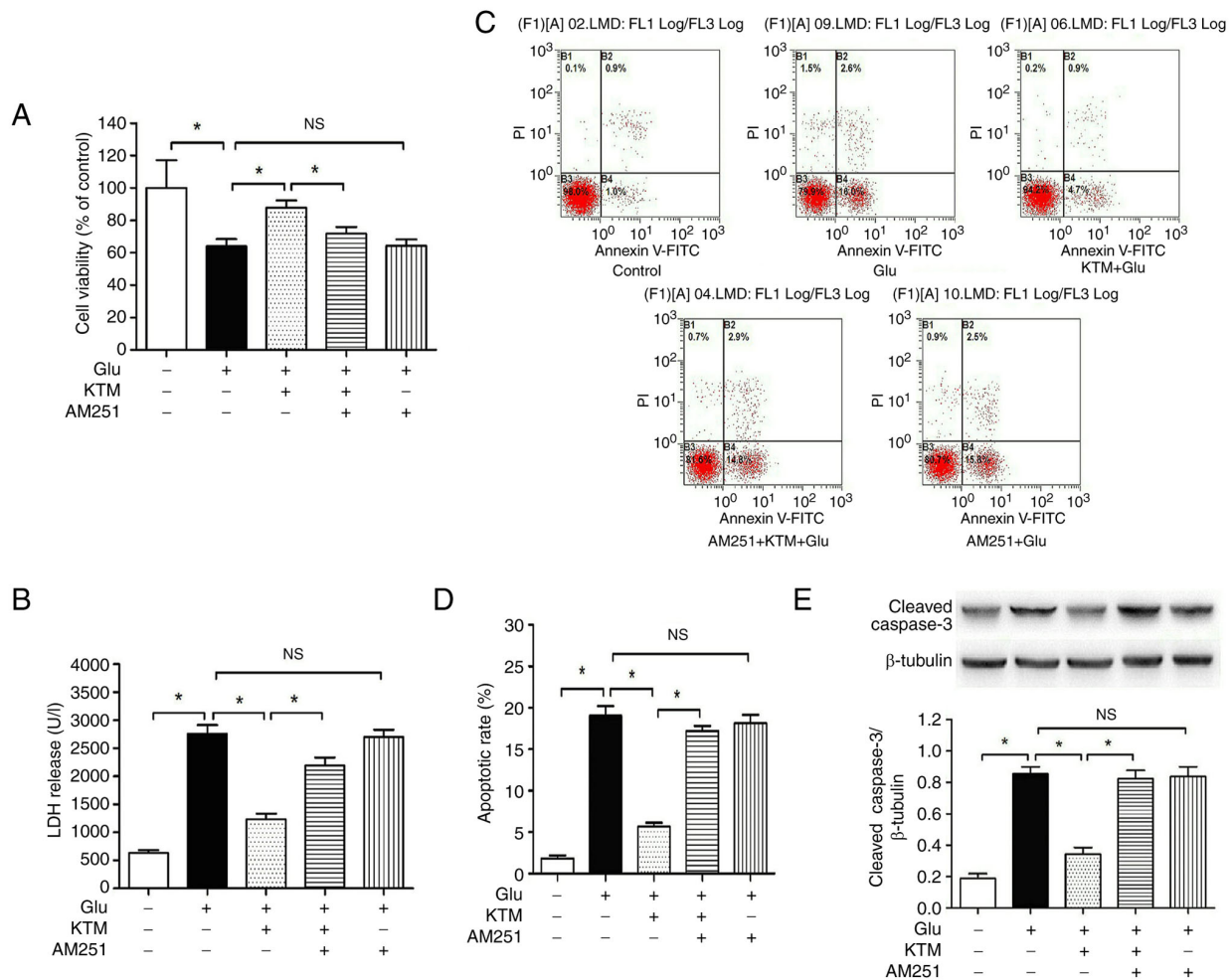
Figure 2. CB1 antagonist AM251 blocks KTM-induced protective effects in HT22 cells treated with Glu. The cells were grouped as follows: i) Control (cultured in drug-free medium); ii) Glu (exposed to the medium with 15 mM Glu), iii) KTM + Glu (the medium with 10 μg/ml KTM and 15 mM Glu); iv) CB1 receptor antagonist AM251 + KTM + Glu (the medium with 10 μM AM251, 10 μg/ml KTM and 15 mM Glu); and v) AM251 + Glu (the medium with 10 μM AM251 and 15 mM Glu). MTT assay and LDH release were used to measure the degree of cell injury level, and flow cytometry was used to assess cell apoptosis rate, and apoptosis protein cleaved caspase-3 was measured by western blotting. (A) AM251 blocked KTM-induced restoration of cell viability (n=10). (B) AM251 blocked KTM-induced inhibition of LDH release (n=8). (C) Cell apoptosis results. (D) AM251 reversed KTM-induced anti-apoptosis effects (n=6). (E) AM251 blocked KTM-induced downregulation of cleaved caspase-3 expression (n=4). *P<0.05. NS, no significance; KTM, ketamine; CB1, cannabinoid receptor 1; Glu, glutamate; LDH, lactate dehydrogenase.

to study the CB1 receptor in KTM-induced neuroprotection. Compared with the cells of control group (Fig. 2), 15 mM glutamate decreased the degree of cell viability (Fig. 2A), and enhanced the LDH level (Fig. 2B), cell apoptosis rate (Fig. 2C and D) and cleaved caspase-3 expression (Fig. 2E) (P<0.05). Co-administration of 10 μg/ml KTM significantly attenuated the glutamate-induced cell injury (Fig. 2A-E); however the presence of the CB1 receptor antagonist AM251 (10 μM), partially blocked the KTM-induced cytoprotection, and AM251 did not bring about notable effects on the glutamate-induced cell injury (P>0.05). According to the flow cytometry results, the right two quadrants indicated early (lower quadrant) and late (upper quadrant) apoptotic cells, respectively, and the rate of apoptotic cells was the sum of the percentage of the lower right and upper right quadrants (Fig. 2C and D).

CB1 antagonist AM251 blocks KTM-induced upregulation of the CB1 receptor in glutamate-treated HT22 cells. Compared with the cells cultured in drug-free medium (Fig. 3A and B),

15 mM glutamate did not cause marked changes in CB1 receptor expression in HT22 cells (P>0.05), and 10 μg/ml KTM significantly upregulated CB1 expression. However, 10 μM CB1 antagonist AM251 (P<0.05) partially reversed the KTM-induced CB1 upregulation (P<0.05); and AM251 did not induce notable effects on CB1 expression, compared with the glutamate-treated cells (P>0.05).

To exclude the potential cytotoxicity caused by KTM or AM251, the cell grouping was as follows (Fig. 3C): i) Control; ii) 10 μg/ml KTM group; iii) KTM + AM251 group and iv) 10 μM AM251 group. After incubation for 24 h, no notable cell injury was observed in the other three groups (P>0.05), compared with that of the control group, indicating that the KTM-induced cytoprotection was via pharmacological effects rather than cytotoxic effects.

KTM blocks glutamate-induced oxidative injury in HT22 cells and cytoprotection is reversed by the CB1 antagonist AM251. A high concentration of glutamate brings about oxidative injury to neuronal cells. In the present study, compared with
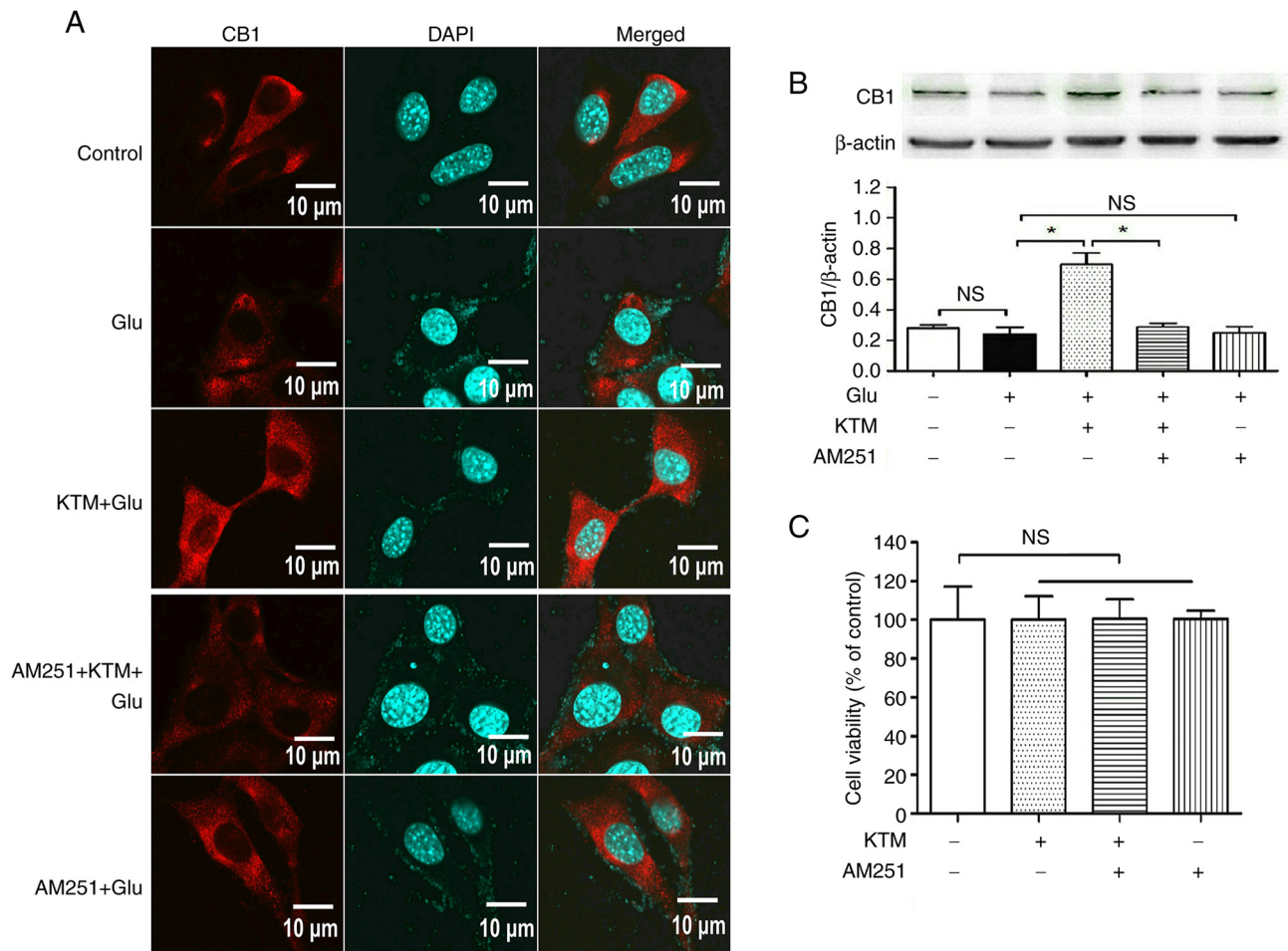
Figure 3. CB1 antagonist AM251 blocks KTM-induced CB1 receptor upregulation in HT22 cells exposed to Glu. CB1 expression level was evaluated by immunocytochemistry and western blotting. Initially, cells were grouped as described in Fig. 2. Then, the cells were grouped as follows: i) Control; ii) 10 µg/ml KTM group iii) 10 µM AM251 group; and iv) KTM + AM251 group. After treatment for 24 h, an MTT assay was used to measure cell viability. (A) Immunocytochemistry results of CB1 protein expression (magnification, x400). (B) Western blotting results of CB1 protein expression (n=4). (C) No obvious cytotoxicity was observed in the presence of AM251 and KTM (n=10). *P<0.05. NS, no significance; KTM, ketamine; CB1, cannabinoid receptor 1; Glu, glutamate; LDH, lactate dehydrogenase.

the control cells, 15 mM glutamate decreased intracellular redox glutathione (Fig. 4A) and CAT (Fig. 4E) levels (P<0.05), and enhanced the GSSG (Fig. 4B) and ROS (Fig. 4C and D) levels. In addition, 10 µg/ml KTM notably blocked the glutamate-induced consumption of intracellular GSH and CAT, and decreased the generation of ROS and GSSG (P<0.05). The CB1 antagonist AM251, however, significantly reversed the aforementioned KTM-induced effects on intracellular antioxidants and oxidants, and AM251 alone did not cause marked influence on the glutamate-induced effects on intracellular GSH, GSSG, ROS and CAT (P>0.05). These findings supported the hypothesis that the CB1 receptor mediates KTM-induced antioxidative injury against glutamate in neurons.

KTM increases intracellular SOD2 levels, but not SOD1 levels. To further explore the KTM-induced antioxidative injury mechanism, intracellular SOD1 and SOD2 levels were measured. A concentration of 10 µg/ml KTM was used to treat the cells. After treatment for 24 h, western blotting and SOD reagent kits were used to evaluate intracellular SOD1 and SOD2 expression levels and activities (Fig. 5A-D). KTM exposure did not alter SOD1 expression and activity (P>0.05);

however, it increased SOD2 expression and activity in HT22 cells (P<0.05), compared with the cells of the control group. As SOD1 is expressed in the cytoplasm, and mitochondria contain SOD2 protein, according to the findings of the present study, it can be inferred that the KTM-induced cytoprotection against glutamate may be via maintaining mitochondrial function and SOD2 levels.

CB1 antagonist AM251 reverses KTM-induced mitochondrial function amelioration, superoxide decrease and SOD2 upregulation. To explore the role of CB1 in KTM-induced cytoprotection, the mitochondrial function, superoxide and SOD2 protein levels were measured. Compared with the control, the mitochondrial complex activities (complex I and IV) and SOD2 expression levels (Fig. 6A, B and E) were significantly reduced in the presence of 15 mM glutamate, and mitochondrial superoxide levels (Fig. 6C and D) were increased. In addition, 10 µg/ml KTM significantly increased the mitochondrial complex activities and SOD2 expression levels, and decreased mitochondrial superoxide levels in the glutamate-treated neuronal cells. AM251 significantly blocked the KTM-induced effects on complex I and IV
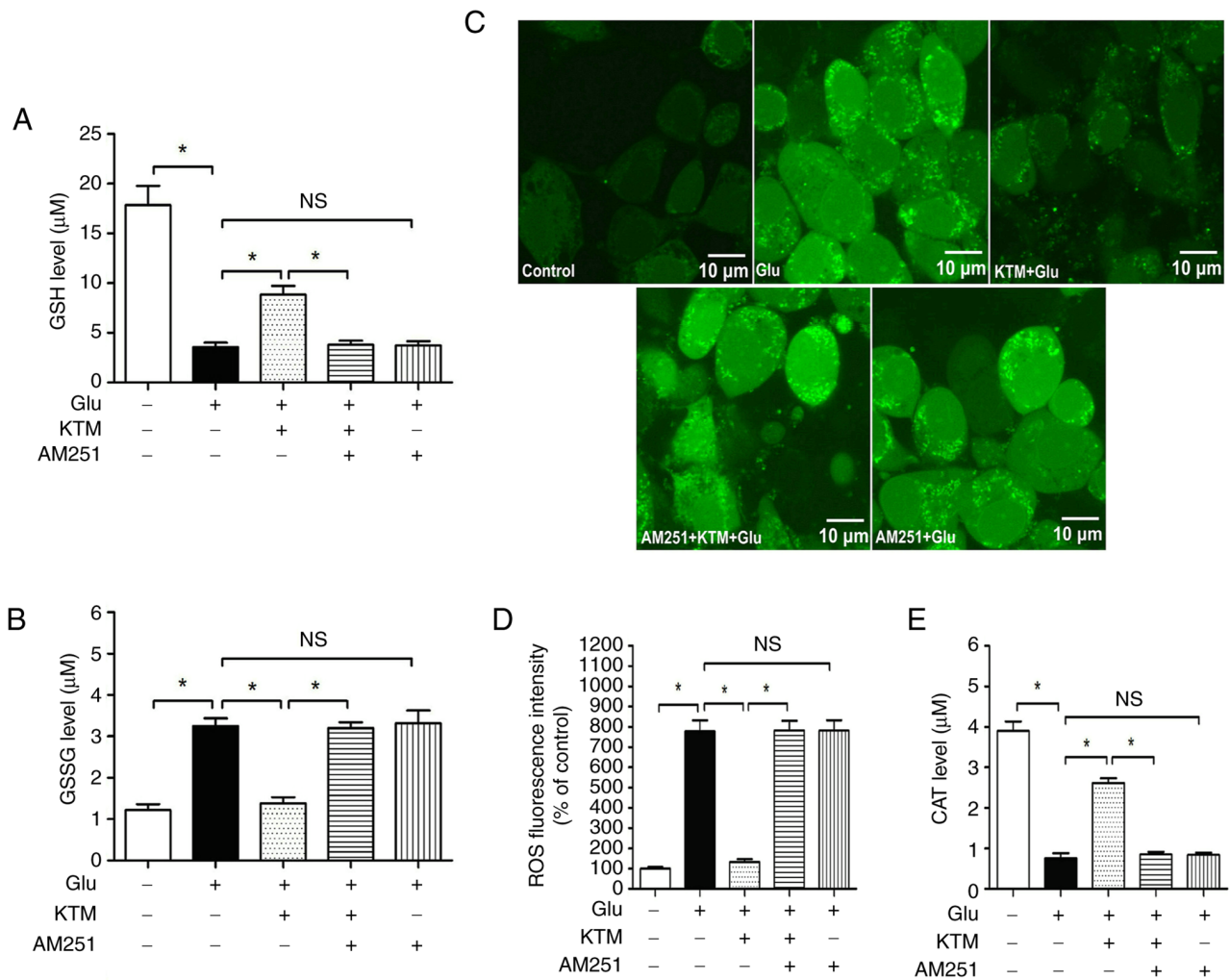
Figure 4. CB1 antagonist AM251 reverses KTM-induced ameliorations of intracellular antioxidants/oxidants. Cells were grouped as described in Fig. 2. Intracellular GSH, GSSG, ROS and CAT levels were measured, and the fluorescence images were captured using a fluorescence microscope. (A) AM251 reversed KTM-induced ameliorations of intracellular GSH levels (n=6). (B) AM251 reversed KTM-induced intracellular GSSG decrease (n=6). (C) Intracellular ROS images (magnification, x400). (D) AM251 reversed KTM-induced intracellular ROS decrease (n=6). (E) AM251 reversed KTM-induced ameliorations of intracellular CAT level (n=6). *$P<0.05$. NS, no significance; GSH, glutathione; GSSG, oxidized GSH; ROS, reactive oxygen species; CAT, catalase; KTM, ketamine; CB1, cannabinoid receptor 1; Glu, glutamate.

levels, and mitochondrial superoxide and SOD2 expression levels ($P<0.05$). AM251 did not cause notable changes in the aforementioned single glutamate-induced oxidative injuries. These findings suggested that KTM-induced cytoprotection against glutamate is mediated by the CB1 receptor through maintaining mitochondrial function.

Discussion

In the present study, HT22 neuronal cells were treated with glutamate to imitate oxidative stress injury in MDD, and it was found that glutamate decreased cell viability and intracellular antioxidants levels, including GSH, CAT and SOD2, and inhibited mitochondrial complex levels. In addition, LDH release, intracellular ROS, GSSG, cell apoptosis, cleaved caspase-3 expression and mitochondrial superoxide levels were increased. However, the presence of KTM significantly decreased the glutamate-induced cell oxidative injury and the effects on the intracellular antioxidant/oxidant levels, and upregulated mitochondrial function and CB1 receptor

expression simultaneously. Co-administration with the CB1 antagonist AM251 notably blocked the KTM-induced cytoprotective effects against glutamate, and downregulated CB1 upregulation. AM251 did not induce marked effects on glutamate-induced oxidative injury. These findings indicated that KTM can decrease glutamate-induced oxidative injury in neuronal cells, and the CB1 receptor may mediate the aforementioned protective mechanisms.

MDD is a common psychiatric condition, with symptoms including prolonged low mood, insomnia and pessimism, and a high risk of suicide (20,21). In the United States, MDD costs the economy $210 billion USD (45% direct losses, 50% workplace losses and 5% suicide-associated losses), causing a great economic load to families of patients and to society as a whole (21). Previous investigations have indicated that a sub-clinical dose of KTM can bring about notable therapeutic effects to treat MDD, and the administration of KTM may also decrease the risk of suicide in patients with MDD (6,8). However, the therapeutic mechanisms of KTM in the treatment of MDD remain unknown. Previous studies
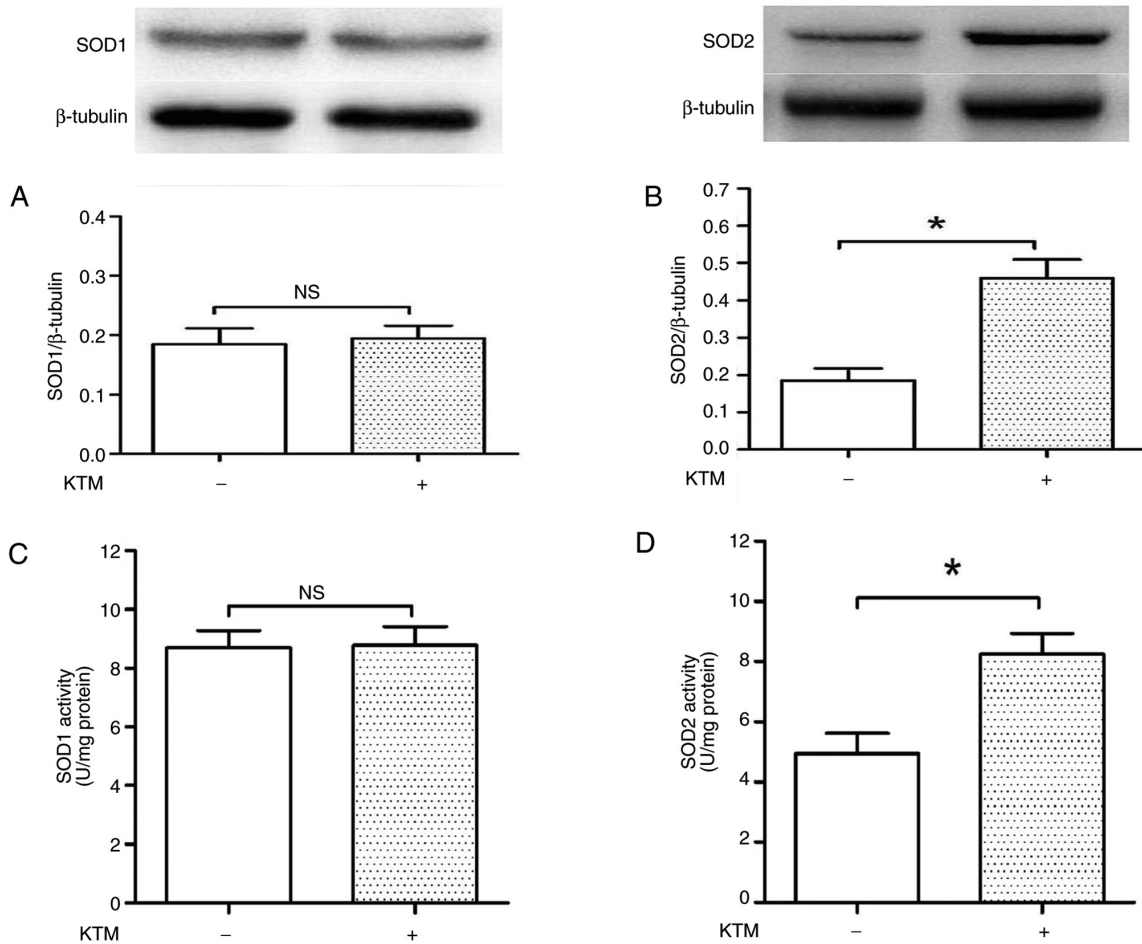
Figure 5. KTM exposure upregulates SOD2 expression and activity, but not SOD1, in HT22 cells. The cells were grouped as follows: i) Normal cultured control and ii) 10 μg/ml KTM group. After treatment for 24 h, SOD1/SOD2 expression levels and activities were measured by western blotting and reagent kits. (A) KTM did not have notable effects on SOD1 expression (n=4). (B) KTM upregulated SOD2 expression (n=4). (C) KTM did not change intracellular SOD1 activity (n=6). (D) KTM enhanced intracellular SOD2 activity (n=6). *P<0.05. NS, no significance; KTM, ketamine; SOD, superoxide dismutase.
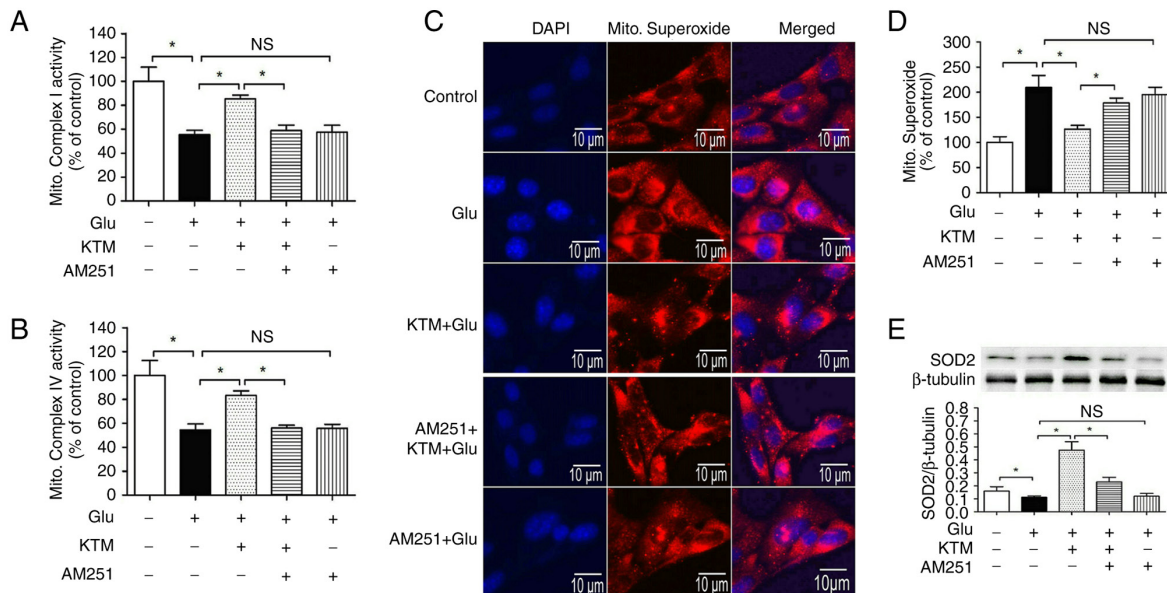

Figure 6. CB1 antagonist AM251 reverses KTM-induced ameliorations of mitochondrial function, superoxide and SOD2 protein levels in glutamate-treated neuronal cells. The mitochondrial function was evaluated according to the mitochondrial complex activities via reagent kits, and mitochondrial superoxide and SOD2 protein levels were measured by immunocytochemistry and western blotting analysis, respectively. (A) Mitochondrial complex I activity (n=6). (B) Mitochondrial complex IV activity (n=6). (C) Mitochondrial superoxide fluorescence staining (magnification, x400). (D) AM251 reversed KTM-induced inhibition of mitochondrial superoxide (n=6). (E) AM251 inhibited KTM-induced upregulation of intracellular SOD2 protein level (n=4). *P<0.05. NS, no significance; KTM, ketamine; SOD, superoxide dismutase; CB1, cannabinoid receptor 1.

have indicated that administering KTM can increase CB1 and CB2 receptor expressions *in vivo*, and that CB receptors may be involved in the anti-MDD effects of KTM (9,10). Moreover, a previous study showed that CB receptors can modulate the psychostimulant effects of KTM in mice (22). At present, two CB receptors, CB1 and CB2, have been discovered in the brain tissue. The CB1 receptor is located in neurons and astrocytes, and the CB2 receptor is expressed in microglial cells and astrocytes (23). Therefore, in the present study, the role of the CB1 receptor in KTM-induced anti-depressive effects was explored.

Previous studies have identified that intracellular antioxidants could be reduced in the pathological mechanisms of MDD, meanwhile, neuronal mitochondrial function may also be decreased (24,25). In patients with MDD, it is reported that mitochondrial ATP production may be inhibited. Moreover, chronic stress could inhibit the activities of mitochondrial complex I, III and IV. This inhibition on mitochondrial complex activity could be reversed by KTM, thus supporting the hypothesis that KTM-induced anti-MDD therapeutic effects may act via attenuating oxidative stress injury (15). Glutamate is an excitatory neurotransmitter in the central nervous system, and high levels of glutamate are commonly used to imitate oxidative stress injury *in vitro* (16). In the pathophysiological processes of MDD, cerebral glutamate concentrations are elevated (26). In the present study, the glutamate exposure decreased intracellular antioxidants, including GSH, CAT and SOD2, and generated oxidants simultaneously, such as GSSH, ROS and mitochondrial superoxide. These findings revealed that glutamate can induce oxidative injury in HT22 cells. During oxidative injury, intracellular GSH can be oxidized into GSSG, therefore the intracellular GSH/GSSG levels are often measured to assess cellular oxidative injury. As mitochondria generate energy for cell metabolism, mitochondrial dysfunction can cause energy (ATP) supply deficiency for cellular activities. In the serum of patients with MDD, the level of the antioxidant vitamin E is lower than that of healthy individuals. In addition, patients with MMD have less mitochondrial DNA copy numbers than that of healthy individuals (13,14). This may explain why mitochondria play an important role in the progression of MDD. Moreover, SOD can be divided into three types including SOD1, SOD2 and SOD3. In animals, SOD1 and SOD3 are in the cytoplasm and intercellular space, respectively, and SOD2 is expressed in the mitochondria (27). As the present study was *in vitro*, SOD1 and SOD2 levels were observed. KTM exposure induced significant upregulations in SOD2 activity and expression compared with SOD1. Therefore, the KTM-induced effects on the mitochondrial functions of HT22 cells were observed. It was found that KTM notably ameliorated the glutamate-induced decrease of mitochondrial complex I and IV activities. Generally, high levels of mitochondrial complex I and IV indicate healthy mitochondrial function (28). In the present study, it was observed that the CB1 receptor antagonist AM251 partially blocked KTM-induced neuroprotection, which supported the hypothesis that the CB1 receptor mediates KTM-induced neuroprotection against antioxidative injury. In fact, in neurons, the CB1 receptor is expressed in the cell membrane and mitochondria, and CB1 receptor upregulation protects neurons and maintains mitochondrial function (28).

In the present study, it was observed that KTM restored the mitochondrial complex activities, upregulated SOD2 expression and activity, and also decreased mitochondrial superoxide levels. As SOD2 is located in the mitochondria, the aforementioned results indicated that KTM-induced neuroprotection may occur through amelioration of neuronal mitochondrial function, which may be mediated by the mitochondrial CB1 receptor. However, further experiments are required to verify cell membrane or mitochondrial CB1-mediated KTM-induced neuroprotection.

The present study investigated the possible therapeutic mechanisms underlying the effects of KTM on MDD, and also showed that the mitochondrial CB1 receptor may be a potential therapeutic target in treating MDD. However, two limitations were identified in the present study. Firstly, a neuronal cell line was used in the present study and it is therefore not known whether similar findings would be discovered when using primary cultured neurons or *in vivo* experiments. Secondly, a CB1 antagonist was used in the present study and this tool uncovered the neuroprotective mechanism of KTM from the perspective of pharmacology. However, whether similar results can be obtained using small interfering RNA or adenoviruses remains unknown. In future studies, the anti-MDD mechanism of KTM will be explored using more tools, and the findings of the present study will be verified in animal experiments.

Finally, in the present study it was found that KTM decreased glutamate-induced oxidative injury in HT22 neuronal cells, and the protection was mediated via the CB1 receptor.

Acknowledgements

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

HB and CW cultured the cells and performed the western blotting and immunocytochemistry. BH and XX performed the statistical analysis. QG designed the study and revised the final version of the manuscript. HB and QG confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Carvalho S, Gonçalves ÓF, Brunoni AR, Fernandes-Gonçalves A, Fregni F and Leite J: Transcranial direct current stimulation as an add-on treatment to cognitive-behavior therapy in first episode drug-naïve major depression patients: The ESAP study protocol. Front Psychiatry 11: 563058, 2020.
2. Cosker E, Moulard M, Schmitt S, Angioi-Duprez K, Baumann C, Laprévote V, Schwan R and Schwitzer T: Portable light therapy in the treatment of unipolar non-seasonal major depressive disorder: Study protocol for the LUMIDEP randomised controlled trial. BMJ Open 11: e049331, 2021.
3. Huang Y, Wang Y, Wang H, Liu Z, Yu X, Yan J, Yu Y, Kou C, Xu X, Lu J, et al: Prevalence of mental disorders in China: A cross-sectional epidemiological study. Lancet Psychiatry 6: 211-224, 2019.
4. Doboszewska U, Wlaź P, Nowak G, Radziwoń-Zaleska M, Cui R and Młyniec K: Zinc in the monoaminergic theory of depression: Its relationship to neural plasticity. Neural Plast 2017: 3682752, 2017.
5. McCall WV, Lisanby SH, Rosenquist PB, Dooley M, Husain MM, Knapp RG, Petrides G, Rudorfer MV, Young RC, McClintock SM, et al: Effects of continuation electroconvulsive therapy on quality of life in elderly depressed patients: A randomized clinical trial. J Psychiatr Res 97: 65-69, 2018.
6. Nikayin S and Sanacora G: Evaluating the role of ketamine/esketamine in the management of major depressive disorder with suicide risk. CNS Drugs 35: 1069-1079, 2021.
7. Roca M, Del Amo AR, Riera-Serra P, Pérez-Ara MA, Castro A, Roman Juan J, García-Toro M, García-Pazo P and Gili M: Suicidal risk and executive functions in major depressive disorder: A study protocol. BMC Psychiatry 19: 253, 2019.
8. Nowak W, Grendas LN, Sanmarco LM, Estecho IG, Arena ÁR, Eberhardt N, Rodante DE, Aoki MP, Daray FM, Carrera Silva EA and Errasti AE: Pro-inflammatory monocyte profile in patients with major depressive disorder and suicide behaviour and how ketamine induces anti-inflammatory M2 macrophages by NMDAR and mTOR. EBioMedicine 50: 290-305, 2019.
9. Pacheco DDF, Romero TRL and Duarte IDG: Ketamine induces central antinociception mediated by endogenous cannabinoids and activation of CB1 receptors. Neurosci Lett 699: 140-144, 2019.
10. Khakpai F, Ebrahimi-Ghiri M, Alijanpour S and Zarrindast MR: Ketamine-induced antidepressant like effects in mice: A possible involvement of cannabinoid system. Biomed Pharmacother 112: 108717, 2019.
11. Pitsillou E, Bresnehan SM, Kagarakis EA, Wijoyo SJ, Liang J, Hung A and Karagiannis TC: The cellular and molecular basis of major depressive disorder: Towards a unified model for understanding clinical depression. Mol Biol Rep 47: 753-770, 2020.
12. Lindqvist D, Dhabhar FS, James SJ, Hough CM, Jain FA, Bersani FS, Reus VI, Verhoeven JE, Epel ES, Mahan L, et al: Oxidative stress, inflammation and treatment response in major depression. Psychoneuroendocrinology 76: 197-205, 2017.
13. Chang CC, Jou SH, Lin TT, Lai TJ and Liu CS: Mitochondria DNA change and oxidative damage in clinically stable patients with major depressive disorder. PLoS One 10: e0125855, 2015.
14. Czarny P, Wigner P, Galecki P and Sliwinski T: The interplay between inflammation, oxidative stress, DNA damage, DNA repair and mitochondrial dysfunction in depression. Prog Neuropsychopharmacol Biol Psychiatry 80: 309-321, 2018.
15. Sharma S and Akundi RS: Mitochondria: A connecting link in the major depressive disorder jigsaw. Curr Neuropharmacol 17: 550-562, 2019.
16. Maher P, van Leyen K, Dey PN Honrath B, Dolga A and Methner A: The role of Ca2+ in cell death caused by oxidative glutamate toxicity and ferroptosis. Cell Calcium 70: 47-55, 2018.
17. Xing Y, Zhang M, Li WB, Dong F and Zhang F: Mechanisms involved in the neuroprotection of electroacupuncture therapy for ischemic stroke. Front Neurosci 12: 929, 2018.
18. Yang S, Hu B, Wang Z, Zhang C, Jiao H, Mao Z, Wei L, Jia J and Zhao J: Cannabinoid CB1 receptor agonist ACEA alleviates brain ischemia/reperfusion injury via CB1-Drp1 pathway. Cell Death Discov 6: 102, 2020.
19. Jia J, Zhang L, Shi X, Wu M, Zhou X, Liu X and Huo T: SOD2 mediates amifostine-induced protection against glutamate in PC12 cells. Oxid Med Cell Longev 2016: 4202437, 2016.
20. Zhang Q, Hong S, Cao J, Zhou Y, Xu X, Ai M and Kuang L: Hippocampal subfield volumes in major depressive disorder adolescents with a history of suicide attempt. Biomed Res Int 2021: 5524846, 2021.
21. Cipriani A, Furukawa TA, Salanti G, Chaimani A, Atkinson LZ, Ogawa Y, Leucht S, Ruhe HG, Turner EH, Higgins JPT, et al: Comparative efficacy and acceptability of 21 antidepressant drugs for the acute treatment of adults with major depressive disorder: A systematic review and network meta-analysis. Focus (Am Psychiatr Publ) 16: 420-429, 2018.
22. Xu W, Li H, Wang L, Zhang J, Liu C, Wan X, Liu X, Hu Y, Fang Q, Xiao Y, et al: Endocannabinoid signaling regulates the reinforcing and psychostimulant effects of ketamine in mice. Nat Commun 11: 5962, 2020.
23. Chen DJ, Gao M, Gao FF, Su QX and Wu J: Brain cannabinoid receptor 2: Expression, function and modulation. Acta Pharmacol Sin 38: 312-316, 2017.
24. Vaváková M, Ďuračková Z and Trebatická J: Markers of oxidative stress and neuroprogression in depression disorder. Oxid Med Cell Longev 2015: 898393, 2015.
25. Giménez-Palomo A, Dodd S, Anmella G, Carvalho AF, Scaini G, Quevedo J, Pacchiarotti I, Vieta E and Berk M: The role of mitochondria in mood disorders: From physiology to pathophysiology and to treatment. Front Psychiatry 12: 546801, 2021.
26. Lener MS, Niciu MJ, Ballard ED, Park M, Park LT, Nugent AC and Zarate CA Jr: Glutamate and gamma-aminobutyric acid systems in the pathophysiology of major depression and antidepressant response to ketamine. Biol Psychiatry 81: 886-897, 2017.
27. Zelko IN, Mariani TJ and Folz RJ: Superoxide dismutase multigene family: A comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. Free Radic Biol Med 33: 337-349, 2002.
28. Ma L, Jia J, Niu W, Jiang T, Zhai Q, Yang L, Bai F, Wang Q and Xiong L: Mitochondrial CB1 receptor is involved in ACEA-induced protective effects on neurons and mitochondrial functions. Sci Rep 5: 12440, 2015.