Multifunctional Molecule, JM-20, Reverses Aluminum Chloride-Induced Memory Impairment and Neuronal Damage in Rats

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disease that worsens with aging. Today, there is a worldwide effort to find new drugs that could delay the onset, slow the progression, or improve symptoms of AD. Oral administration of aluminum to rodents recapitulates some pathological alterations observed in AD, being considered a convenient tool for modeling and testing the efficacy of new therapeutics. Our previous studies have shown that JM-20, a dihydropyridine and benzodiazepine hybrid molecule protected memory in an environment with cholinergic dysfunction, high oxidative stress, hyperactivation of acetylcholinesterase (AChE) enzyme, and mitochondrial damage produced by scopolamine. In order to gain further insight into the effects on JM-20 on AD pathology, we evaluated the protective effects of JM-20 after chronic $AlCl_3$ administration to rats, and assessed several types of episodic memory alterations and associated-pathological mechanisms, including mitochondrial dysfunction, AChE hyperactivity, inflammation, and apoptosis-related proteins. We used behavioral tasks to test spatial, working an emotional- associative memory, as well as molecular, enzymatic and histological assays to evaluate selected biochemical parameters. Our study showed that JM-20 prevented memory decline alongside with the inhibition of aluminum-induced alterations of oxidative parameters, and increase of AChE activity and of tumor necrosis factor alpha (TNF-α) levels. JM-20 also preserved anti-apoptotic proteins and protected against axonal and neuronal damaged in the hippocampus and prefrontal cortex. Altogether, our findings expanded our understanding of the ability of JM-20 to preserve essential types of memory in rats under neurotoxic conditions, and suggest its potential capacity to counteract etiological factors of AD by breaking the progression of key neurodegeneration-associated steps in a rat model of the disease.

Highlights

- JM-20 improves different type of memory in aluminum animal model of dementia.
- Mitochondrial and antiapoptotic mechanisms are suggested as central way of JM-20 prevent memory damage.
- JM-20 has potential as a therapeutic candidate for the treatment and management of Alzheimer’s disease.

Introduction

The number of patients with some type of dementia has increased with numbers at 50 million towards the end of the 2019, a figure set to increase to 152 million by 2050. Alzheimer's disease (AD) is the most common form of dementia, accounting for approximately 60% of all types of dementia, which will prove to be a great financial burden to society in the future [1]. AD is a complex and multifactorial disorder, for which aging is the main identified risk factor, whereas Ca^{2+} dyshomeostasis, oxidative stress, energy deficits, and mitochondrial dysfunction are all emerging as potential biochemical factors [2-4]. Currently, symptomatic treatment for the cognitive deficits in AD targets either cholinergic (with

acetylcholinesterase inhibitors) or glutamatergic transmission (through N-methyl-D-aspartate (NMDA) receptor antagonists, e.g., memantine). However, treatments with a single symptomatic agent have shown insufficient efficacy [5-7]. Therefore, it is imperative to find new drugs that could delay the onset, slow progression, or improve symptoms of AD, to save lives and the economy. It has been extraordinarily difficult to develop new therapies. The failure rate has been over 99% and no new treatment has been approved since 2003 [8]. Attempts to develop disease-modifying therapies have had a 100% failure rate [9]. Every trial, however, presents opportunities to learn and improve the drug development process [10,9]. In this context, small molecules with multitarget effects provide a promissory strategy on smart drug design focused to target various components in AD pathology [11].

JM-20 (3-ethoxycarbonyl-2-methyl-4-(2-nitrophenyl)-4,11-dihydro-1H-pyrido[2,3-b][1,5] benzodiazepine), is a novel hybrid molecule with a 1,5 benzodiazepine fraction covalently linked to a dihydropyridine ring, forming an innovative entity with proven multisite neuroprotective activity [12,11,13-15]. JM-20 protects mitochondrial functionality under several damage conditions both *in vitro* and *in vivo* studies, and acts as a strong antioxidant compound. Notably, this molecule prevents death and improves the neurological function in experimental animal models of ischemic stroke and Parkinson´s disease without amnesic or toxic effects [12,11,13-15]. In addition, other pharmacological effects were detected as direct or indirect action of JM-20 in the complex scenery of the pathological models of AD. Oral administration of JM-20 prevents memory impairment, in episodic memory and in acquisition-consolidation processes against acute scopolamine treatment [16]. Drawing from all these positive antecedents, we hypothesized that this molecule could revert and prevent neurotoxic chronic damage that involves more complex long-term mechanisms.

Aluminum is a neurotoxic metal that contributes to the progression of several neurodegenerative diseases including AD by combining several mechanisms. Numerous investigations have showed that oral administration of aluminum to rodents recapitulate several pathologic mechanisms involved in AD including cognitive decline. The neurodegenerative effects involve the formation of intraneuronal filamentous aggregates that are tau positive, followed by oxidative stress, neuroinflammation, and apoptosis [17-19]. Apoptosis is a type of programmed cell death mediated by diverse molecules such as p21, p38, mitogen-activated protein kinase (MAPK), p53, caspases 2, 3, 8 and 9, BCL-XS and Bax apoptosis inducing factors, Par-4 (prostate apoptosis response 4), while Bcl-XL and Bcl-2B (cell lymphoma/leukemia-2 protein) inhibit apoptosis by a number of different mechanisms. Apoptosis is essential for normal homeostasis and elimination of dysfunctional cells. Balance between the antagonistic effects of the proapoptotic and antiapoptotic members has been involved in the maintenance of the cell integrity. Mitochondria are cytoplasmic organelles that regulate both metabolic and apoptotic signaling pathways; mitochondrial failure is associated with early events in the pathogenesis of aging-related neurodegenerative disorders and neuronal cell loss [20,21].

Thus, the present study was designed to explore the impact of JM-20 on aluminum-induced memory impairment in rats. We hypothesized that treatment with JM-20 is capable of rescuing mitochondria from aluminum-induced neurotoxicity, preventing apoptotic signals that lead to neuronal death and thus

preventing the impairment of multiple types of memory in this AD model. Here, we observed that JM-20 acted through the inhibition of AChE hyperactivity, also providing mitochondrial and oxidative protection, normalization of TNF-α levels, and the preservation of anti-apoptotic signals.

Materials And Methods

Compounds and reagents

JM-20 was synthesized, purified, and characterized as previously reported [22], in Analytic Chemistry Laboratory of CIDEM. All general chemicals used were purchased from Sigma-Aldrich (St. Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) kits for tumor necrosis factor-α (TNF-α) and interleukin-1β (Il-1β) was purchased from PeproTech (Rocky Hill, NJ, USA). Western blot antibodies Akt, phospho-Akt (Ser473), GSK-3β, phospho-GSK-3β (Ser9), caspase-3, cleaved caspase-8 (Asp387) were purchased from Cell Signaling Technology, Inc. (USA).

Animals and ethics statement

For the experiments 12 weeks old male Wistar rats (220±5 g) obtained from the National Center for the Production of Laboratory Animals (Mayabeque, Cuba) were used. All animals were kept in standard animal cages under conventional laboratory conditions (12 h light/dark cycle, 23 ± 2°C), with access to food and water *ad libitum*. The rats were handled for 7 days before behavioral testing by removing them from the home cage for a few seconds and then returning them to the cage. Experimental behavioral procedures were performed during the light phase. All procedures were performed in compliance with institutional and international regulations (Animal Care Committee from CIDEM, Havana, Cuba. National Institutes of Health Guide No. 80-23, 1978) of protection of animals, and all efforts were made to minimize the number and suffering of animals used in this study.

Experimental design

Previous studies have shown that 500 mg/kg $AlCl_3$ (i.g) induced memory impairments in adult rats [23,24]. $AlCl_3$ (Sigma Aldrich, Canada) was dissolved in distilled water and administered daily (500mg/kg; 4ml/kg) by oral gavage (i.g) for 30 consecutive days prior to the trials and then until the end of the behavioral examinations. JM-20 (2 or 8 mg/kg; 4 ml/kg) was suspended in a carboxymethylcellulose (CMC; vehicle) solution 0.05 % w/v. Rats were treated daily with a single dose of JM-20 (2 or 8 mg/kg; 4 ml/kg; p.o.) starting on 15th day after the beginning of $AlCl_3$ treatment and until the end of the study. The dose range was selected based on previous experience [22]. To eliminate possible interactions, administration between these two compounds was staggered 30 minutes.

Animals were randomly divided into 5 experimental groups (n=10 animals per group): (1) non-damaged control group treated with vehicle (CMC and distilled water), (2) non-damaged control group treated with 8 mg/kg JM-20, (3) damaged control group treated with 500 mg/kg AlCl3 plus JM-20 vehicle (CMC) (4) group treated with $AlCl_3$ plus 2 mg/kg JM-20 and (5) group treated with $AlCl_3$ plus 8 mg/kg JM-20.

Behavioral studies

Morris water maze (MWM), Y-maze (Ym) of spontaneous alternation, novel object recognition (NOR), and passive avoidance tests were performed to monitor potential change in different types of episodic memory. Behavioral studies started after 30 days of $AlCl_3$ administration (i.e. after 15 days of JM-20 treatment). Different sets of animals were used for the MWM, Ym of spontaneous alternation, novel object recognition, and passive avoidance tests, to maintain similar period of time between the battery of test (30 first days after $AlCl_3$ administration).

Y-maze test. Working memory was examined using the spontaneous alternation modality of Y-maze (Ym) test. The Ym apparatus was made of plastic with three equal arms (55 cm × 20 cm × 20 cm) extending from a central platform with an angle of 120° [25]. The test consisted of a single trial allowing the animal to explore the three arms for 8 min. An arm entry was counted when the hind paws of the rat were completely within the arm. Alternation was defined as successive entries into the three arms on overlapping triplet sets (i.e. A, B, C or B, C, A, etc.). The number of maximum spontaneous alternation was then the total number of arms entered-2 and the percentage is calculated as the ratio of actual to possible alternations (defined as the total number of arm entries-2). The entries in each arm of the maze were recorded using a video-recording camera. After each trial, the apparatus was cleaned with ethanol 40 % to reduce olfactory cues.

Morris water maze test. Spatial learning and memory were investigated using MWM paradigm in a circular pool, 150 cm in diameter and 60 cm high, filled with water (24±1 °C) to a depth of 30 cm. The pool was divided into four quadrants and a movable escape platform (10 cm diameter) located in the center of a fixed quadrant. Eight points equally distributed along the perimeter of the tank were served as starting locations. The apparatus was located in a room with well-ordered extra-maze clues (clues on each four cardinal points) that remained constant throughout the experiment. Rats were allowed training sessions (acquisition trial) of 60 s, four times each day for four consecutive days. During each acquisition trial, animals were left free to find the platform in the designated quadrant. Once the rat located the platform, or if a subject failed to reach the platform within 60 s, it was placed on the platform for 30 s. On the fifth day, a probe test (retrieval trial) was performed where the platform was removed, and each rat was allowed to explore the pool for 60 s. The time spent by the animal in the target quadrant searching for the hidden platform and the number of crossings on the platform site, was regarded as indexes of retrieval [26].

Novel object recognition test. Memory of object recognition was explored with NOR test, performed as described previously [27] with some modifications. Animals were habituated to the empty chamber (rectangular chamber, 65 × 45 × 45 cm) [28] for 5 min in two habituation sessions 60 minutes apart, 24 hours before the beginning of the behavioral test. The NOR test consisted of two five-minute sessions, trials separated by an intertrial interval of 60 min. In the first trial (acquisition or training session), animals were exposed to two identical objects (F, familiar object) located 10 cm from the sides of the chamber and 25 cm apart. During the second trial (T2, retention or testing trial), rats were exposed to one F and to

a novel object (N) of a different shape and color from F. Each rat was placed into the chamber between the two objects facing the opposite direction. Rats were monitored using a video-recording camera. The amount of time exploring F and N objects during T1 and T2 was recorded, and then total time exploring F and N, and discrimination index ((N - F)/(N+F)) was calculated. After each trial, the apparatus and objects were cleaned with 40 % ethanol to reduce olfactory cues.

Passive avoidance test. Emotional associative memory was explored by passive-avoidance response, using a suitable modified protocol [29]. An avoidance response apparatus (UGO Basile, Germany) with two independent compartments, a light shock-free zone (L-SFZ) camera and a dark shock zone (D-SZ) camera, both with a wire grid floor and a connection door between cameras, was used. The test rat was placed on the L-SFZ for the first 30 s for habituation, then the door was opened. When the animal stepped off this L-SFZ on the next to 90s and enter to the D-SZ, it received a shock (0.5 mA) for 3 s (acquisition or training trial). 24 h after training, the animal was placed on the L-SFZ and the door to D-SZ was opened (retention or testing trial). Retention parameters (step-through latency) were measured for 300 s and compared for different treatments. Animals which had not entered to D-SZ on training session were rejected of this test.

Acetylcholinesterase activity on brain homogenate

After behavioral testing, the rats were euthanized, their brains were promptly removed and the hippocampus (HO) and prefrontal cortex (PC) were dissected and stored at -80°C. Samples were homogenized and protein quantification was determined by Lowry´s method [30]. AChE activity was measured in aliquots of PC and HO using Ellman´s method (n=9 per group) [31]. The assay was performed in 96-well microtiter plates using the Ellman's reagent DTNB (5,5'-dithio-bis(2- nitrobenzoic acid, 10 mM), 50 µL of homogenate and 10 µL of ATChI (acetylcholine iodide, 20 mM), final volume 200 µL. The absorbance was read at 412 nm during 20 min at 1 min interval. The AChE activity was expressed as µmol of (ATCh) iodide hydrolyzed per min per mg protein [16,32].

Mitochondrial assays

Isolation of brain mitochondria. Rat brain mitochondria were isolated as previously described [33] with minor modifications. Briefly, rats were decapitated and their brains were rapidly removed and placed into 10 mL of an ice-cold isolation buffer (1 mM) ((mannitol 225 mM, sucrose 75 mM, K-EGTA 10 mM), bovine serum albumin 0.1 % and K-HEPES (4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid) (10 mM, pH 7.2). The forebrains were cut into small pieces using surgical scissors, which were extensively washed in isolation buffer. The tissue was manually homogenized in a glass Dounce homogenizer with a loose-fitting pestle and a tight-fitting pestle. The homogenate was centrifuged for 3 min at 2000 x g in a Hettich centrifuge (Germany), Rotanta 460R. The resulting supernatant was centrifuged for 8 min at 12, 000 x g. The pellet was resuspended in a 10 mL isolation buffer containing 20 µL of digitonin 10%, which was used to release synaptosomal mitochondria, and centrifuged for 10 min at 12 000 x g. The supernatant and the upper light layer of the pellet were discarded, and the dark pellet was resuspended in an isolation buffer without EGTA. This homogenate was then centrifuged for 10 min at 12, 000 x g. The supernatant

was discarded, and the final pellet was gently washed and resuspended in an EGTA-free isolation buffer at an approximate protein concentration of 30-40 mg/mL. The entire procedure was performed at 4°C. The respiratory control ratio (state 3/state 4 respiratory rate), measured using succinate (5mM) as the substrate [15], was greater than 4.

Standard incubation procedure. Rat brain mitochondria (0.5 mg/mL) were energized with potassium succinate (5 mM) (plus 2.5 μM rotenone) in a standard incubation medium consisting of sucrose (125 mM), potassium chloride (65 mM) and HEPES–KOH (10 mM), pH 7.4 at 4 °C [32].

Mitochondrial membrane potential. The mitochondrial membrane potential (ΔΨ) was determined spectrofluorimetrically using safranine O (10 μM) as a probe at 495/586 nm excitation/emission wavelengths. These assays were performed in the presence of EGTA (0.1 mM) and K_2HPO_4 (2 mM) [11,16,32].

Mitochondrial swelling. Mitochondrial swelling induced by calcium (Ca^{2+}) plus inorganic phosphate (Pi) was estimated spectrophotometrically from the decrease in the absorbance at 540 nm measured using the spectrophotometer [12]. Brain mitochondria (0.5 mg/mL) were added to the standard incubation medium supplemented with Ca^{2+} (50 μM) plus Pi (2 mM).

Production of reactive oxygen species. Reactive oxygen species (ROS) were monitored spectrofluorimetrically using Amplex red (1 μM) (Molecular Probes, OR, USA) and horseradish peroxidase (1 UI/mL) at 563/587 nm excitation/emission wavelengths [11]. Amplex Red oxidation in the presence of extra mitochondrial horseradish peroxidase bound to hydrogen peroxide (H_2O_2) generates resorufin, a highly fluorescent compound, with a 1:1 stoichiometry [34]. Brain mitochondria (0.5 mg/mL) were added to the standard incubation medium supplemented with the ROS probe plus EGTA (100 μM). Standard curve obtained by adding known amounts of H_2O_2 to the standard incubation medium in the presence of the reactants (amplex red and horseradish peroxidase) was linear up to 2 μM. The slope of the increase in fluorescence was converted to the amount of H_2O_2 (10 fluorescence units represent 80pmol of H_2O_2).

Antioxidant enzymes and MDA levels

Antioxidant enzyme activities (catalase (CAT), superoxide dismutase (SOD), total sulfhydryl (T-SH) groups and the extent of lipid peroxidation in the brain (amount of malondialdehyde (MDA) were assayed as we previously reported [32]. Protein content was measured using Lowry's method with bovine serum albumin as a standard.

Western blot analysis

Western blotting was carried out according to previously published protocols, with slight modification [35]. In brief, the HO and PC tissues were homogenized with the RIPA lysis buffer containing a protease and phosphatase inhibitor cocktail (Sigma-Aldrich, USA), and centrifuged at 12, 000 × g for 30 min. The samples were standardized in sample buffer (62.5 mM Tris-HCl pH 6.8, 2% (*w/v*) sodium dodecyl sulfate

(SDS), 5% β-mercaptoethanol, 10% (*v/v*) glycerol, 0.002% (*w/v*) bromophenol blue), boiled fat 95 °C for 5 min and separated using SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and incubated with rabbit anti-phosphorylated Akt (Ser473; P-Akt, 1:1000, Cell Signaling Technology, Danvers, MA, USA), anti-Akt (1:1000; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-phosphorylated GSK-3β (Ser9; P- GSK-3β, 1:1000, Cell Signaling Technology, Danvers, MA, USA), anti-GSK-3β (1:1000; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-Bcl-2 (1:1000; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-Bax (1:1000; Cell Signaling Technology, Danvers, MA, USA) rabbit anti-caspase 3 (1:500; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-caspase 8 (1:500; Cell Signaling Technology, Danvers, MA, USA), and anti-β-tubulin (1:1000; Cell Signaling Technology, Danvers, MA, USA) antibodies, overnight at 4°C. The membranes were then incubated with goat anti-rabbit IgG horseradish peroxidase (1:5000; Cell Signaling Technology, USA) for 120 min. Following the detection of chemiluminescent bands (Immobilon™ Western chemiluminescence kit, #P90720, Millipore), they were quantified using Image J software 6.0. Data are expressed as percentage of control values after normalization using β-tubulin as internal standard.

Inflammatory Response: TNF-α and IL-1β levels

The TNF- α levels and IL-1β were measured in brain homogenates (HO and PC) using a rat ELISA kit from Peprotech. The results were expressed in nanograms per milliliter. The average minimum sensitivity of the ELISA kit detection is 0.4 ng/mL of cytokines [36].

Histopathological studies

After perfusion, whole brains of five rats, randomly selected from each group, were allowed to fix overnight in formalin 4 % and then were cut in coronal sections, keeping the HO and PC for the examination. The atlas of Paxinos and Watson was used to identify each brain region [37]. Subsequently, the tissues were embedded in paraffin and processed for hematoxylin and eosin (HE) staining and cut into 4μm coronal sections, always taking the first three cuts from each region. These samples were observed in a Leica optical microscope (Microsystems, Germany). Neuronal damage was assessed in areas CA1, CA2, CA3 and dentate gyrus of the HO and on PC. Atrophy, nuclear pyknosis, dark cytoplasmic staining or absence of neurons were considered as markers of neuronal degeneration. Additionally, axonal damage specifically based on the appearance of partially or completely demyelinated axons, as well as axonal atrophy was calculated and expressed as a percentage of the total axons for the region studied. In order to perform the analysis of histological damages at the PC level, the average number of neurons and affected axons was counted in three fixed areas of each cerebral hemisphere by two independent experimenters, who were blind to the group treatments. The results were expressed as percentage of damaged neurons with respect to the total neurons of each area.

Statistical analysis

Statistical analysis was performed by the GraphPad Prism 7.0 program (GraphPad Software Inc., USA). All data are presented as mean ± standard error of the mean (SEM) and their normality and

homoscedasticity were checked by D'Agostino-Pearson normality test and Bartlett's test, respectively. One-way analysis of variance (ANOVA) was performed, followed by Tukey multiple comparison test. A value of p <0.05 was considered significant.

Results

JM-20 rescues recognition, emotional-associative and spatial memory

NOR test was used to evaluate recognition memory; passive avoidance test for emotional associative memory, and spontaneous alternation Ym and MWM tasks to assess spatial memory, all of which are intimately associated with the hippocampal and cortical activity.

In the NOR test, discrimination index in $AlCl_3$-exposed group treated with vehicle were significantly lower when compare to control group (control group: 0.73 ± 0.249; $AlCl_3$-treated group: 0.27 ± 0.225, P<0.001), suggesting $AlCl_3$-induced cognitive impairment. In contrast, damaged animals treated with JM-20 8mg/kg showed significantly higher discrimination index ($AlCl_3$/JM-20 8 mg/Kg: 0.77 ± 0.116, P<0.001) than $AlCl_3$-exposed group (Fig. 2a). Control (non-damaged) animals treated with JM-20 8 mg/Kg (JM-20 8 mg/Kg: 0.69 ± 0.066, P<0.001) showed similar discrimination index to vehicle-treated control group (Fig. 2a). JM-20 2 mg/Kg ($AlCl_3$/JM-20 2 mg/Kg: 0.126 ± 0.232, P>0.05) exerted no protective effect (Fig. 2a).

In the passive avoidance test, results revealed the $AlCl_3$-induced emotional-associative memory impairment. Control group showed a significantly higher latency to enter into the dark compartment (approximately 300 s, maximum evaluation time) than the AlC_3-treated group (control group: 273.8 ± 25.56 s; $AlCl_3$-treated group: 73.25 ± 32.26 s, P<0.001) (Fig. 2b). Noteworthy, JM-20 8mg/kg also reverted the effects of $AlCl_3$ on emotional-associative memory ($AlCl_3$/JM-20 8 mg/Kg: 246.0 ± 78.75, P<0.001), with similar values to control group (P>0.05) (Fig. 2b). Treatment with JM-20 2 mg/Kg to $AlCl_3$-exposed rats ($AlCl_3$/JM-20 2 mg/Kg: 78.00 ± 16.51) showed no protective effect (Fig. 2b).

The decrease of spontaneous alternation (Ym test) in the AlC_3-treated group regarding control (undamaged) group (control group: 72.02 ± 8.84; $AlCl_3$-treated group: 47.70 ± 3.49, P<0.001) evidenced that treatment with $AlCl_3$ also affected the spatial working memory (Fig. 2c). Treatment with JM-20 (2 and 8 mg/kg) preserved working memory, with values closed to control rats and significantly different to $AlCl_3$-exposed group ($AlCl_3$/JM-20 2 mg/Kg: 61.32 ± 11.39, P<0.05; $AlCl_3$/JM-20 8 mg/Kg: 66.54 ± 7.29, P<0.001) (Fig. 2c).

By the other side, in MWM we evaluated the spatial reference memory during a training phase (acquisition), followed by the evaluation phase (retrieval trial). We note that during the acquisition period, the escape latency was significantly lower in the group treated with $AlCl_3$ plus JM-20 8mg/kg when compared to those receiving vehicle, starting on the second day (Fig. 2d). Interestingly, JM-20

administration afforded an improvement in reaching the platform with escape latency approaching the normal values during the second and third day of training (Fig. 2d). Additionally, during the retrieval trial $AlCl_3$-treated rats that received JM-20 (2 and 8 mg/kg) spent more time (sec) in the target quadrant ($AlCl_3$/JM-20 8 mg/Kg: 28.26 ± 7.487, P<0.001; $AlCl_3$/JM-20 2 mg/Kg: 26.23 ± 6.894, P<0.05) when compare to damaged rats treated with vehicle ($AlCl_3$-treated group: 17.10 ± 3.650), and similar to control group (27.85 ± 8.674). JM-20 also significantly increased the mean number of crossing ($AlCl_3$/JM-20 8 mg/Kg: 7.7 ± 2.31, P<0.001; $AlCl_3$/JM-20 2 mg/Kg: 6.6 ± 1.65, P<0.05), as compared with $AlCl_3$ treated rats ($AlCl_3$-treated group: 3.0 ± 2.45) and similar to control values (control group: 7.0 ± 1.70) (Fig. 2 e, f). Treatment with JM-20 to non-damaged rats (non-$AlCl_3$) did not modify any of these behavioral parameters regarding control animals (no $AlCl_3$- or JM-20-treated) (Fig. 2a-e).

JM-20 inhibits $AlCl_3$-induced hyperactivation of AChE in hippocampus

The effect of JM-20 on the AChE activity from HO and PC samples was analyzed to evaluate cholinergic alterations after chronic $AlCl_3$ exposition (Fig.3a, b). Results showed that $AlCl_3$ group exhibited a significant rise in AChE activity in the HO when compared with control animals (no $AlCl_3$ or JM-20 treated), (control group: 0.50 ± 0.16; $AlCl_3$-treated group: 0.94 ± 0.19, P<0.01). However, treatment with JM-20 (8 mg/kg) prevented the $AlCl_3$-induced increase of enzymatic activity in HO ($AlCl_3$/JM-20 8 mg/Kg: 0.69 ± 0.33, P<0.05; $AlCl_3$/JM-20 2 mg/Kg: 1.01 ± 0.31, P>0.05) (Fig.3a). In the PC, neither the $AlCl_3$ exposure nor the JM-20 treatment had significant effects on AChE activity (control group: 0.588 ± 0.205; $AlCl_3$-treated group: 0.725 ± 0.229, $AlCl_3$/JM-20 8 mg/Kg: 0.723 ± 0.240, $AlCl_3$/JM-20 2 mg/Kg: 0.643 ± 0.101, P>0.05) (Fig. 3b).

JM-20 stimulates antioxidant response against $AlCl_3$-induced oxidative stress in hippocampus and prefrontal cortex

Considerable evidence suggests there are beneficial effects of antioxidants on brain degeneration and dementia. In this sense, enzymes such as SOD, CAT, and GPx can determine the clearance of free radicals. Table 1 shows the effect of JM-20 on oxidative stress parameters after chronic $AlCl_3$ administration. To evaluated whether JM-20 confers protection against $AlCl_3$-induced oxidative damage we assessed T-SH content and the main antioxidant enzymatic defenses, SOD and CAT, in homogenates of HO and PC tissues. SOD activities remained unalterable in $AlCl_3$ treated rat (Table 1), similar to undamaged controls. However, we note a significant increase of CAT activity, in both HO and PC, as well as lower levels of T-SH, in $AlCl_3$-treated rats. These effects were reversed by the treatment with JM-20. JM-20 (8 mg/kg) maintains the enzymatic activity and levels of T-SH similar to controls, showing a potent protective effect against oxidative events induced by the daily treatment with $AlCl_3$ (Table 1).

In addition, we measured MDA formation in HO and PC tissue, which is a biomarker of lipid peroxidation. There was a significant increase in MDA concentrations in $AlCl_3$-treated rats when compared to control (vehicle or JM-20-treated) animals (in HO and PC (Table 1). Thus, animals exposed to $AlCl_3$ for 30-days

that received treatment with JM-20 (8 mg/kg), prevents MDA increase concentrations in both cerebral regions. However, JM-20 (2 mg/kg) treated animals did not show significant protection.

JM-20 protects against mitochondrial dysfunctions

Mitochondria are central on energy production, even when the astrocytes provide important support while synaptic transmission is ongoing. The stability and dynamic of the membrane potential, levels of H_2O_2, and mitochondrial swelling are suitable for detecting damage at mitochondrial level. Thus, we observed an increase in the ΔΨm dissipation in $AlCl_3$-treated group (83 %) compared with control animals (Fig. 4a, $P<0.001$), as well as a strong increase in mitochondrial ROS production upper 25% (Fig. 4b, $P<0.001$) and a significant mitochondrial swelling (139 %, Fig. 4c, $P<0.001$). While, JM-20 8 mg/kg on $AlCl_3$ treated animals, exhibited a significant protection of mitochondrial ΔΨm dissipation (33 % compare to control, Fig. 4a, $P>0.05$), lower mitochondrial ROS production (3 % compare to control, Fig. 4b, $P>0.05$) and high but not complete swelling inhibition (70 % of controls, Fig. 4c, $P<0.05$). 2 mg/kg JM-20 on $AlCl_3$-treated animals did not confer significant protection against $AlCl_3$-impaired mitochondrial functionality. Treatment with 8 mg/kg JM-20 to control animals (no $AlCl_3$-treated) did not show differences with respect to control group ($P>0.05$).

JM-20 reduces TNF-α in hippocampus and prefrontal cortex

Astrocytes and microglia actively participate in the inflammatory response releasing anti- and pro-inflammatory cytokines and chemokines. It has been well established that microglial activation and elevated tumor necrosis factor-alpha (TNF-α) are critically involved in cognitive deficit. As shown in Figure 5, the levels of TNF-α were significantly higher in the HO and PC of $AlCl_3$ treated rats (in HO, control group: 41.44 ± 1.41 pg/mg and $AlCl_3$-treated group: 52.5 ± 2.90 pg/mg, $P<0.01$; in PC, control group: 41.41 ± 0.92 pg/mg and $AlCl_3$-treated group: 51.8 ± 1.63 pg/mg, $P<0.05$) (Fig. 5a-d). While, $AlCl_3$-damaged animals treated also with JM-20 8 mg/Kg maintained the levels of TNF-α similar to undamaged control group (HO: $AlCl_3$/JM-20 8 mg/Kg: 41.97 ± 1.58 pg/mg, $P<0.01$; PC: $AlCl_3$/JM-20 8 mg/Kg: 38.99 ± 2.11 pg/mg, $P<0.05$ compared to $AlCl_3$ group). We did not detect any alteration in the IL-1β levels caused by interventions ($AlCl_3$ or JM-20 treatments or both) in neither HO nor PC (Fig. 5c-d).

Antiapoptotic effects of JM-20 against aluminum-induced neuronal death

We analyzed the PI3K cell-signaling pathway by measuring its effects on the phosphorylation of proteins Akt and GSK-3β by western blot (Figure 6a, b). Next, we investigated the protective role of JM-20 treatment over $AlCl_3$-induced neuronal death mediated by signals downstream GSK-3β phosphorylation and via apoptosis activation.

No differences were observed in Akt activation (p-Akt) between undamaged groups and the $AlCl_3$-treated group or JM-20-treated group (Figure 6a, 7a). Interestingly, $AlCl_3$ exposure decreased the phosphorylation of GSK-3β (Figure 6b, 7b) in HO and PC, suggesting the activation of this pro-apoptotic protein. This

effect was counteracted by JM-20 treatment (2 mg/kg and 8 mg/kg), which indicates that JM-20 maintained increased the levels of phosphorylated GSK-3β in both regions.

Balance of Bcl-2 family members, including antiapoptotic proteins (such as Bcl-2 and Bcl-xL) and proapoptotic members (such as Bad, Bid, and Bax/Bak) regulates apoptosis through the maintenance of mitochondrial integrity [38]. In this study, the Bax/Bcl-2 ratio indicates that in the $AlCl_3$-group treated with vehicle apoptotic signaling were favored ratio >1; significantly higher than in the undamaged group) in HO and PC (Figure 6c, h). In the $AlCl_3$-group treated with 8mg/kg JM-20 the Bax/Bcl-2 ratio in both regions was significantly lesser compared with $AlCl_3$- damaged group that received vehicle (CMC). Western blotting analysis also revealed that $AlCl_3$ exposition caused the increase of pro-apoptotic caspase-3 and caspase-8 levels, effect that was prevented by JM-20 treatment in both cerebral regions (Fig. 6d-e; 7d-e). Further, when compared to control group, the results revealed that $AlCl_3$ exposure up-regulated hippocampal and cortex GSK-3β but not Akt on the way to the activation, and increased Bax levels and key caspases involved in extrinsic and intrinsic apoptosis pathway. Interestingly, JM-20 succeeded to counteract $AlCl_3$-induced neurotoxicity and cellular death and gave greater protection, since it was not observed high levels of caspase 3.

JM-20 restored histopathological alterations in keys anatomical structures

To confirm the irreversibility of the apoptosis induced the $AlCl_3$-treated group, and the protective role of JM-20, it would be relevant to evaluate some markers of late apoptosis, like loss of cell membrane integrity, DNA fragmentation or cytomorphological alterations [39]. In this sense, we move to a histological approach, with the aim to verify final apoptotic stages. The biochemical results of the current study were further confirmed by the histological examination of the HO and the PC. As mentioned earlier, hematoxylin-eosin (H&E) staining were performed to detect neurons in degeneration in the brain sections. The microscopic examination of the sections obtained from the normal group revealed an intact architecture of the tissues with normal histological appearance and distribution of the neuronal cells (Figure 8 a-d). In contrast, the HO of the $AlCl_3$-treated group showed degenerative changes and atrophy of the neuronal cells and apoptosis (control group: 5.00 ± 2.24 % of neurons showing signs of degeneration respect to total; $AlCl_3$-treated group: 27.29 ± 9.48 %, $P<0.001$) (Figure 8a, c). Similarly, the PC of the $AlCl_3$-treated group presented neuronal atrophy and cells in advanced state of death (control group: 8.14 ± 4.02 % respect to total; $AlCl_3$-treated group: 32.71 ± 11.53 %, $P<0.001$) (Figure 8 b, d). JM-20 (8 mg/kg) administration to $AlCl_3$-treated rats attenuated the pathological degenerative changes and signs of death in the HO (8.29 ± 3.18 % respect to total, $P<0.001$; Figure 8 a, c) and PC areas (10.86 ± 2.61 % respect to total, $P<0.001$; Figure 8 a, c). JM-20 at 2 mg/kg exerts a smaller effect compared with the quadruple of the dose (8mg/kg). JM-20 administration to non-$AlCl_3$ treated rats did not alter the normal appearance of the brain sections (JM-20: 5.14 ± 2.27 % respect to total, $P<0.001$ respect to $AlCl_3$ group) (Figure 8 a, c). These observations indicate that JM-20 mitigated the histopathological and biochemical alterations in keys anatomical structures involved in the memory process.

Discussion

AD is a degenerative brain disease that becomes worse with the age. Symptoms occur because neurons and glial cells in specific regions of the brain involved in thinking, learning, and memory have been damaged. Over time, symptoms tend to increase and start interfering with individuals' ability to perform daily activities, and after nine years of symptom onset, patients die [9, 10]. Therefore, it is imperative to find new drugs that could delay the onset, slow progression, or improve symptoms of AD. Such neurotoxic effects were observed in the present study by the prolonged intake of $AlCl_3$, which significantly prompt neuropathological alterations in HO and PC of rats that are consistent with several reports [43,17,44-46]. After treatment with JM-20, a high level of neuroprotection was observed, confirming our preliminary hypothesis that it provides neuroprotection. Treatment with JM-20 rescued mitochondria from aluminum-induced neurotoxicity, preserved anti-apoptotic proteins and, consequently, restored multiple types of memory and neurobiological status in this AD model. These effects were accompanied by oxidative, AChE and mitochondrial stabilization of level and function, whereas neuronal viability at biochemical and histological level were verified. Overall, JM-20 therapy showed potential to counteract different aspects of $AlCl_3$-induced pathological mechanisms.

Considering that cognitive decline and the lack of visuospatial memory are both clinical symptoms of AD [40], the deficit of learning and spatial memory have been widely reported in studies with transgenic mice and pharmacological model of AD [42,42]. Considering this, we used the AD model induced by AlCl3 neurotoxicity. The deposits of $AlCl_3$ are involved in several signals in the brain, specially related to neuroinflammation [47], mitochondrial disorders [48], cellular death [44], and behavioral signs of dysfunction in working, spatial and emotional-associative memory. Moreover, the hippocampus is one of the most vulnerable cerebral structures; suffering from a decline in synaptic function in this structure can lead to dementia [49].

As previously reported by others, our study indicates that chronic exposure to $AlCl_3$ significantly impaired spatial memory in rats, according to MWM and Y maze tests results, and also the contextual memory as revealed in the passive avoidance test [50-52]. In the Y maze test, $AlCl_3$-treated rats had impaired working memory, failed to make correct alternations in a new environment. In the MWM, $AlCl_3$ over loaded rats also showed less capacity to retrieve and retain the location of hidden platform reflecting the inability to encode and remember the spatial information even after several days of training. In the passive avoidance test, $AlCl_3$-treated rats do not remember the aversive stimulus past 24 h and enter the dark chamber associated with electric shock earlier in comparison to control rats that could remember and avoid the dark chamber. JM-20 co-administration after 15 days of $AlCl_3$ treatments, until day 30, reversed aluminum induced memory deficits, which indicate its memory protecting effect. Our previous work denotes that JM-20 reversed the memory deficits induced by scopolamine, an indication of cognitive protection mediated by this molecule, but in a transient model of dementia, useful to detect JM-20 activity against a very fast cholinergic dysfunction (thirty minutes after injection)[16]. In the present study,

JM-20 also showed a protective effect but against chronic aluminum exposure, more similar to clinical conditions.

AChE, a cholinesterase that hydrolyses acetylcholine (ACh), is considered one of the biomarkers of cholinergic function in the brain. AChE has high sensitivity to exogenous factors including aluminum [53-55]. Increased AChE activity stimulates the breakdown of ACh, leading to cholinergic system abnormal function. As cholinergic transmission contributes to learning, memory and cognition and it is closely related to short- and long-term memory [56], the degree of impairment in these synapses correlates with the severity of dementia in patients [57]. Aluminum is a well-known potent cholinotoxin [58], which can alter the blood brain barrier to trigger changes in the cholinergic transmission [59]. In addition, it significantly elevates AChE activity directly [60], through the interaction between aluminum and AChE peripheral sites, modifying the secondary structure of the enzyme [61,62]. Here, in line with these previous studies, our results showed considerably elevated AChE activity in $AlCl_3$ group when compared to control groups [52,63,64]. However, co-administration of JM-20 to $AlCl_3$-intoxicated rats restored physiological AChE activity. Thus, JM-20 can reduce the negative effects of AlC_3 in the brain mediated by AChE hyperactivity, as well as ameliorated the learning and memory impairment by $AlCl_3$ exposure. It is noteworthy that JM-20 has inherent anti-AChE activity properties. Previously we have demonstrated using *in vitro* and *in silico* studies that JM-20 has a strong AChE inhibitory activity ($IC_{50} \approx 200$ nM) [16,65]. Therefore, JM-20 administration can directly inhibit AChE activity in the Hippocampus, restoring memory dysfunction because cholinergic neurons densely innervate the hippocampus, mediating the formation of episodic memory [66,56].

The cognitive impairment found in AD has been also associated with oxidative damage and the subsequent unbalance on the endogen antioxidant system in the brain, one of the major causes of brain aging. Previous studies have reported elevation of brain oxidative stress markers due to the impairment of the antioxidant enzyme system in neurodegenerative diseases, a feature that can be induced by the presence of aluminum [68, 69]. Regarding this experimental evidence, our study revealed that $AlCl_3$-treated rats showed significant decrease of CAT activity (antioxidant enzymes activities), while increasing MDA production in the HO and PC, corroborating with the hypothesis of impaired antioxidant system in this AD model. Treatment with JM-20 was able to counteract both the decrease in antioxidant activity and the accumulation of oxidative stress markers, maintaining levels similar to healthy-control rats, while SOD activity was unaltered. Despite these indirect antioxidant effects, JM-20 has intrinsic antioxidant potential. Its reduction potential of -0.72 V makes it an excellent electron acceptor, capable of preventing the generation of ROS at the mitochondrial level [15]. Recent results confirmed these effects on scopolamine-induced cholinergic and memory impairment and in rotenone-induced neurotoxicity in experimental model of Parkinson's disease [16]. Collectively, these data suggests that the effects of JM-20 in decreasing oxidative stress may involve not only its direct antioxidant effects, but also an indirect antioxidant action favoring a cellular antioxidant environment.

Considerable evidence suggests there are beneficial effects of antioxidants on brain degeneration and dementia. A large body of evidence demonstrates a real oxidative environment on an AD brain over a long progression period [70-72]. In fact, recent evidence states that oxidation products could act as biomarkers in some neurodegenerative diseases, such as lipid peroxidation markers 4-hydroxynonenal and MDA, that were are identified in the cortex and hippocampus in AD patients [73]. The oxidative damage may be related to a decrease in antioxidants and repair systems. In this sense, enzymes such as SOD, CAT, and GPx (glutathione peroxidase), can determine the clearance of free radicals [74]. Brains with decreased SOD and the CAT activities observed in hippocampal neurons and glial cells may stimulate an overproduction of superoxide and H_2O_2 molecules, alongside with excessive NO production (via iNOS activity) cause severe cellular injury [75,76,74]. Moreover, SOD released from astrocytes is hypothesized to protect GSH by reducing the production of superoxide [77]. In addition, the increase in GPx activity may induce decreased levels of GSH, the main antioxidant defense of the brain, which may contribute to excitotoxicity because it may increase the vulnerability to oxidation [78] and may induce an excessive inflammatory response in age-related neurodegenerative diseases [79].

Several investigations indicate that the mitochondrion is a target for AD. It has been hypothesized that the dysfunction in this organelle may be at the heart of the progression of AD itself and is primarily involved in ROS production in the CNS [80,81]. In addition, brains with decreased hippocampal SOD and the CAT activities may stimulate an overproduction of superoxide and H_2O_2 molecules, causing severe cellular injury [74-76]. After 38 days of treatment, isolated brain mitochondria of $AlCl_3$ treated rats showed high membrane potential dissipation, increased ROS production and significant swelling. In this sense, our work verified the role of JM-20 on mitochondrial dysfunction rescue, after a period of $AlCl_3$ exposure. JM-20 was able to reverse mitochondrial malfunction and preserve this organelle by maintaining membrane potential, normalizing H_2O_2 production and partially preventing mitochondrial swelling. We did not detect signs of damage at this level on ex *vivo* preparations, and normal rates of O_2 production were measured. These result are in correspondence with our previous reports to evaluating JM-20 effects on ischemic, Parkinson and in AD transient's model, were detected even protection of mitochondrial anatomy [15,16]. Importantly, the structural characteristics of JM-20: small molecular weight (404.14 g/mol), cationic, and lipophilic molecule with a log P of 3.46 (partition coefficient of a molecule to predict solubility and permeability), allow it to reach the mitochondrial compartment promptly making this organelle a potential pharmacological target for it actions. Taking all together, we hypothesized that mitochondrial preservation is the major mechanism of JM-20 neuroprotective actions, which resulted in better performances in memory tests by treated animals.

Having higher metabolic rates and energy demands, neuronal cells depends a lot on mitochondrial function. Mitochondria supply ATP (adenosine triphosphate) to the cells (via oxidative phosphorylation), synthesize key molecules, and respond to oxidative stress as well as in apoptosis/survival signals. Mitochondrial production of ATP supports synapse assembly, generation of action potentials, and synaptic transmission, all of this essential for cognitive function [82,83,84]. Numerous works describe the central role of damaged mitochondria on the neurodegenerative pathophysiology, as a background of

oxidative stress is perpetuated by the installation of mitochondrial dysfunction. Damaged mitochondria produce excess superoxide and hydroxyls radicals, H_2O_2, and protein-lipid peroxidation, which disrupts mitochondrial DNA and cause an imbalance in mitochondrial respiratory chain, Ca^{2+} homeostasis, excitotoxicity, membrane permeability, apoptosis and mitochondrial defense systems [80,85]. Those are notable causes of the propagation of neuronal dysfunction, triggering neurodegeneration with the consequent loss of cerebral functions [80,85]. In this sense, a mitoprotective drug is a potential candidate for treating dementia signs and its progression, in order to avoid the ordinary course of neurodegeneration [83,86].

Neurodegenerative processes are associated with induction of neuroinflammation and release of cytokines, its propagation and its consequences, ending in dysfunction and cell death [87]. Patients suffering with AD exhibit increased levels of cytokines in serum, brain, and cerebrospinal fluid [89,90]. In addition, oxidative/nitrosative stress can also induce an inflammatory response by increasing TNF-α levels [88]. These and other sings of neuroinflammation are the basis of neuroinflammatory hypothesis of AD late onset [87]. TNF-α induces astrocyte and microglial activation and is closely associated with increases in another proinflammatory cytokines, such as IL-1β. In the present study, we investigated $AlCl_3$ effect on brain homogenates and detected high levels of TNF-α on HO and PC of $AlCl_3$ treated rat, but we did not observe abnormal levels of IL-1β. In these conditions, JM-20 avoided the increase of TNF-α and exerted a strong protection, both in the HO and PC, in rats treated during 38 days with $AlCl_3$.

The early release of TNF-α is essential in priming microglial cells and innate immunity to effectively resolve initial damage, and its absence delays activation of microglial cells which leads to an exaggerated and nonspecific activation, amplifying secondary damages [91]. However, TNF-α is considered a pro-inflammatory factor that is synthesized by microglia, astrocytes, and some populations of neurons [92,93], with receptors in the same cellular population, exhibiting direct and indirect actions on neurons [94-96]

TNF-α can bind to two specific receptors: TNFR1 (tumor necrosis factor receptor 1), with an intracellular death domain, and TNFR2 (tumor necrosis factor receptor 2), with a higher affinity and mostly involved in neuroprotection but also contributes to cell injury mediated by NF-□B and caspase-3 activation leading neuronal death [97]. Such activities amplify the oxidative stress that is deleterious to biochemical and cellular processes, in cyclic events of damage. The result is chronic neuroinflammation, dysregulation of cellular functions, and further neuronal death. In memory function, it was described that TNF-α overproduction also regulates synaptic plasticity region dependently, for example, inhibition of long-term potentiation in the hippocampus [98,99]. Therefore, we propose that JM-20 mitigates neuroinflammation through the attenuation of cytokine synthesis and release, potentially increasing neuronal survival and preserving mnemonic process. This mechanism may be an indirect result of JM-20 mitoprotective effects.

In both AD pathology and in $AlCl_3$-induced toxicity, we have several signs of injury: the mitochondria, which are important sensors and executioners in the cell's decision to live or die by intrinsic pathways, the

expression of TNF-α that lead to neuronal death in long-lasting conditions mediated by extrinsic signals and a continuous, not resolved, oxidative stress. In this sense, we quantify levels of essential key mediators of survival and death signals (Akt, GSK-3β, Bax/Bcl-2 ratio, caspase 8), as well, as the executer of apoptosis cellular death (caspase 3). Our results pointed out a significant increase in the inactivation of GSK-3β against $AlCl_3$-treated rats, both in the hippocampus and prefrontal cortex. In the brain, GSK-3β regulates many crucial cellular processes, acting as a key switch that controls numerous signaling pathways [reference]. Different evidences support the role of GSK-3β on beta amyloid (Aβ) and tau neurotoxicity, mediated by mechanism of direct production and regulation (e.g. inhibiting ADAM activity, or in tau protein hyperphosphorylation (Ptau) together CDK-5 kinase action) [reference]. Increased GSK-3β activity has been used to model events occurring in AD, interventions that exacerbate cognitive impairments, and neuropathology in rodent models of AD, like in aluminum rat model of AD [102]. Even when we do not evaluate Aβ and Ptau formation, several reports mention the putative capacity of aluminum to produce these reactive species, keys on AD neuropathology [103]. Then, we suppose that the increased levels of GSK-3β on $AlCl_3$ group is the beginning of a neurotoxic pathway that it is possibly acting in the APP processing and multiple tau phosphorylation. In addition, concerning with synaptic transmissions is well documented that neuronal GSK-3β overexpression causes a decrease in postsynaptic density number and volume in hippocampal granule neurons [104], a phenomenon that maybe related to cognitive impairment and altered LTP generation [105]. At that point, JM-20 possibly stopped these slowly by consistent process that lead damage, and prevent cognitive decline.

Apoptosis might also trigger an adaptive immune response, associated to the release of cell death associated danger signals (cDAMPs) including (but not limited to): ATP, nucleic acids the non-histone nuclear DNA-binding protein high mobility group box 1 (HMGB1), cytokines like type I interferon (IFN) chaperones like calreticulin (CALR), heat shock protein family and reactive oxygen species (ROS) [100].

Hippocampal and prefrontal cortex homogenates under $AlCl_3$ effects revealed the subsequent impact in different subsystems leading to apoptotic signaling. Values of Bax/Bcl-2 ratio, and levels of caspase 3 and caspase 8 reflect the relative cells status in the hippocampus and prefrontal cortex and indicate irrevocable death of brain cell precedents of $AlCl_3$-treated rats control group. In contrast, treatment with JM-20 preserves at least a few target pathways as oxidation/reduction balance, energetic provision mediated by the mitochondria, physiological non-toxic activity levels of AChE for memory and cognitive processes contributing to brain restoration and non-deleterious process resulting on degeneration and cellular death. After 1 month of JM-20 daily doses, pro-apoptotic mediators of intrinsic and extrinsic pathways and executers markers were similar to healthy group: stable and balanced. At this point, aware that an entire cascade of intermediaries should be quantified, we were led to believe that JM-20 is a molecule able to prevent neuronal death under neuropathological alterations similar to that in AD in humans. Nevertheless, we do not discard the occurrence of other immunogenic and programmed cell death modalities like necroptosis in $AlCl_3$ treated group, which might be modulated by JM-20. Indeed, a previous report provided the first *in vivo* evidence for a role of RIP3 protein in TNF-α-induced toxicity of hippocampal neurons, demonstrating that TNF-α promotes RIP3-MLKL-mediated necroptosis of

hippocampal neurons bypassing ROS accumulation and calcium influx supporting this hypothesis [105]. Evaluation of these markers could contribute to expand the molecular effector mechanisms elicited by JM-20 in the context of $AlCl_3$-induced alterations. To confirm the irreversibility of the apoptosis induced by the $AlCl_3$-treated group, and the protective role of JM-20, it would be relevant to evaluate some markers of late apoptosis, like loss of cell membrane integrity, DNA fragmentation or cytomorphological alterations [39].

To associate JM-20 neuroprotective effects with histological analysis, we evaluated neuronal population by H&E. In the $AlCl_3$ group, a large number of signs of neuronal death and cell loss were detected, whereas JM-20 8 mg/kg protects more than 95% of neurons on HO and PC regions. The results supported that the previous mechanism described for JM-20, mediated memory protection also stopped the degenerative process leading to dysfunction and death, reported in final states of dementia.

In conclusion, based in this and our previous reports, JM-20 has potential as a novel candidate for treating AD condition, acting in key pathological mechanisms of neurodegeneration and preserving structural architecture of brain structures that are essential for cognitive functions. The current results do not cover the full spectrum of possible mechanisms of memory dysfunction and neuronal death; thus, other studies should be performed.

Abbreviations

AD, Alzheimer's disease; **$AlCl_3$**, Aluminum chloride; **ATP**, adenosine triphosphate; **Bcl2**, B cell leukemia/lymphoma 2; **Bax,** Bcl-2-associated X protein; **GSK-3β**, glycogen synthase kinase-3 beta; **Akt**, serine threonine kinase or protein kinase B; **PI3K**, phosphoinositide-3-kinase; **IL-1β,** Interleukin-1β; **MWM,** Morris water maze; **Ym,** Y maze; **TNF-α,** tumor necrosis factor alpha; **NMDA**, N-methyl-D-aspartate; **CAT**, catalase; **SOD,** superoxide dismutase; **GSH**, reduced glutathione; **T-SH,** total sulfhydryl groups; **NO,** nitric oxide; **iNOS,** inducible nitric oxide synthase; **Gpx,** glutathione peroxidase

Declarations

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Availability of Data and Material

All data generated or analyzed during this study are included in this published article.

Ethics Approval

All experimental protocols were reviewed by the Ethics Committee for Animal Experimentation of Center for Pharmaceuticals Research and Development and National Guidelines, carried out according to the Guidelines for Animal Experimentation registered in Resolution No.64/13 (CICUAL) (Center for the State Control of Drugs, Medical Equipment and Devices, Cuba).

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Authors' contributions

Maylin Wong-Guerra: designed the research, performed experiments, analyzed the data and wrote original draft. Yanay Montano-Peguero, Ramírez-Sánchez J, Javier Jiménez-Martin, Luis Arturo Fonseca-Fonseca, Daniela Hernández-Enseñat, Yasmine Nonose, Odalys Valdés, Yaquelin Ortiz-Miranda, Gretchen Bergado and Gilberto Pardo-Andreu: performed experiments and analyzed the data. Adriano Martimbianco de Assis, Roberto Menéndez Soto del Valle, Tiago Fleming Outeiro, Tania Carmenate, Diogo O Souza and Alejandro Saúl Padrón-Yaquis: designed the research, organized the project, financial support and reviewed the paper. Abel Mondelo-Rodriguez: JM-20 chemical design, synthesis and characterization. Yanier Nuñez-Figueredo: designed the research, organized the project, wrote original draft and corresponding author.

Conflicts of interest/Competing interests

The authors declare that they have no conflict of interest.

Consent to Participate

Not applicable

Consent for Publication

The authors declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere. We confirm that the manuscript has been read and approved by all named authors.

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Tables

Table 1. Effect of JM-20 on brain SOD and CAT activities, T-SH and MDA levels in $AlCl_3$ treated rat.

A Hippocampus	Control	JM-20 8mg/kg	$AlCl_3$	$AlCl_3$ JM-20 2mg/kg	$AlCl_3$ JM-20 8mg/kg
SOD U/min/mg protein	17.04 ± 6.415	15.68 ± 2.71	14.46 ± 2.294	16.37 ± 2.13	14.72 ± 4.325
CAT U/min/mg protein	29.62 ± 11.20*	30.42 ± 6.396*	46.42 ± 14.90	33.58 ± 3.43	23.33 ± 11.21**
T-SH nmol/mg protein	14.30 ± 1.24*	13.47 ± 1.83*	7.72 ± 0.48	10.13 ± 1.58	13.51 ± 1.178*
MDA nmol/mg protein	1.32 ± 1.09**	1.506 ± 1.21**	4.15 ± 1.84	1.39 ± 1.696**	1.79 ± 1.045*

A Prefrontal Cortex	Control	JM-20 8mg/kg	$AlCl_3$	$AlCl_3$ JM-20 2mg/kg	$AlCl_3$ JM-20 8mg/kg
SOD U/min/mg protein	14.55 ± 2.60	13.01 ± 4.45	13.70 ± 3.38	10.85 ± 2.15	10.28 ± 0.63
CAT U/min/mg protein	15.96 ± 4.16**	16.77 ± 3.50*	29.87 ± 5.6	19.01 ± 4.72	17.98 ± 5.35*
T-SH nmol/mg protein	14.43 ± 1.28**	12.57 ± 1.38*	7.32 ± 0.78	10.22 ± 1.04	13.09 ± 1.49*
MDA nmol/mg protein	4.44 ± 1.54**	4.56 ± 1.81**	7.92 ± 2.42	3.65 ± 1.60**	4.05 ± 1.39*

Values represent the mean ± SEM (n = 9 per group). Different letters indicate statistically significant between groups: P <0.05. * P <0.05, ** P <0.01 **, P <0.001 compared with the $AlCl_3$ group. CAT: catalase; SOD: superoxide dismutase; T-SH: total sulfhydryl; MDA: malondialdehyde.

Figures

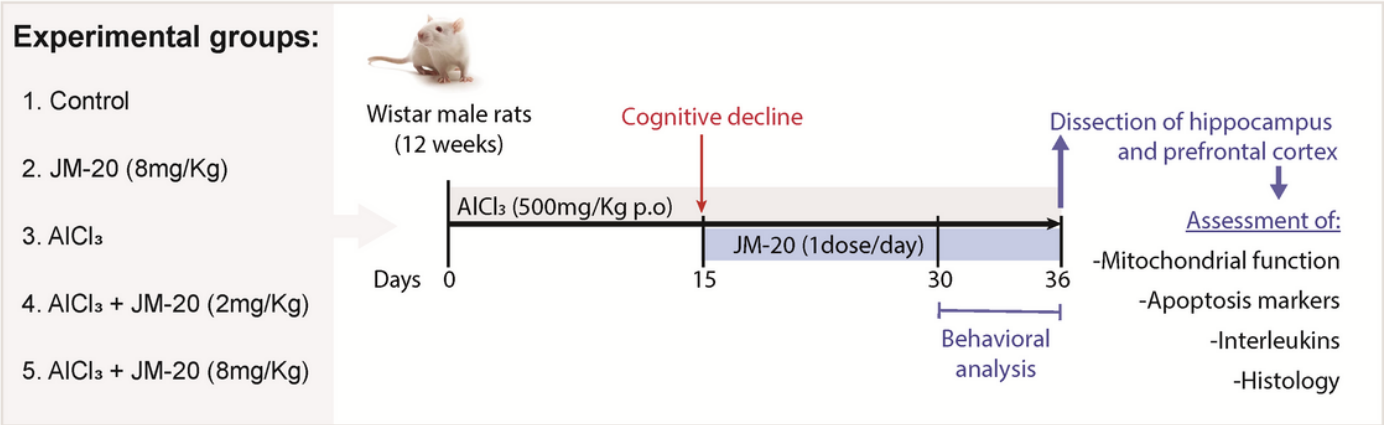

Figure 1

Schematic representation of the experimental design.

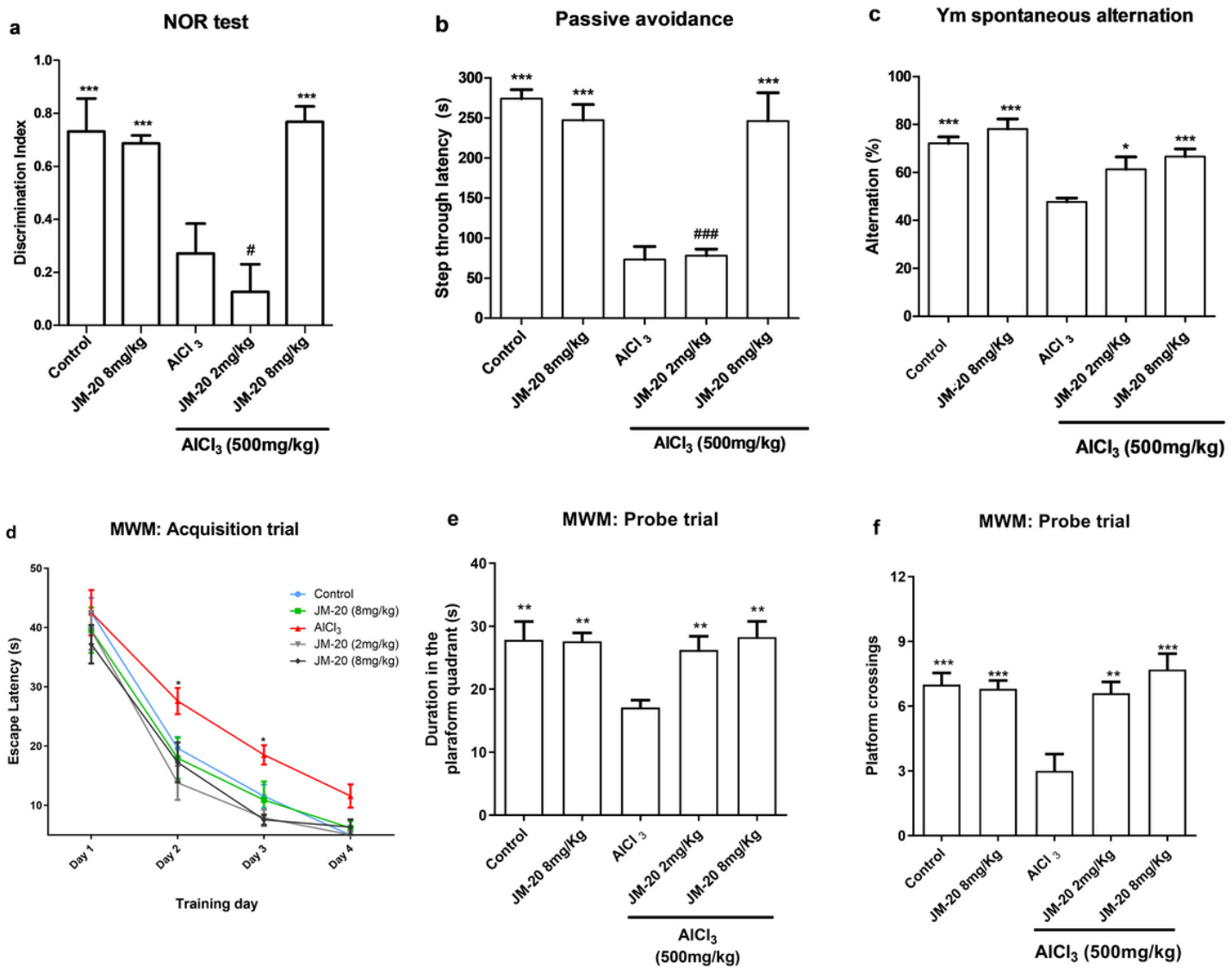

Figure 2

Effect of AlCl3 and JM-20 on learning and memory in rats performing novel object recognition test (a), step through passive avoidance (b), Y-maze (c) and Morris water maze in acquisition (d) and probe (e-f) trials. AlCl3 500 mg/kg i.g or vehicle was administered 30 days daily and during behavioral test. JM-20 (2 mg/kg or 8 mg/kg) started on 15th day after AlCl3, administered daily and during behavioral test. Data are represented as mean ± SEM (n = 9, per group), ANOVA and post hoc Tukey Multiple Comparison Test were used for statistical analysis. * P <0.05, ** P <0.01, *** P <0.001 compared to the AlCl3 group and # P<0.05 compared to the normal group.

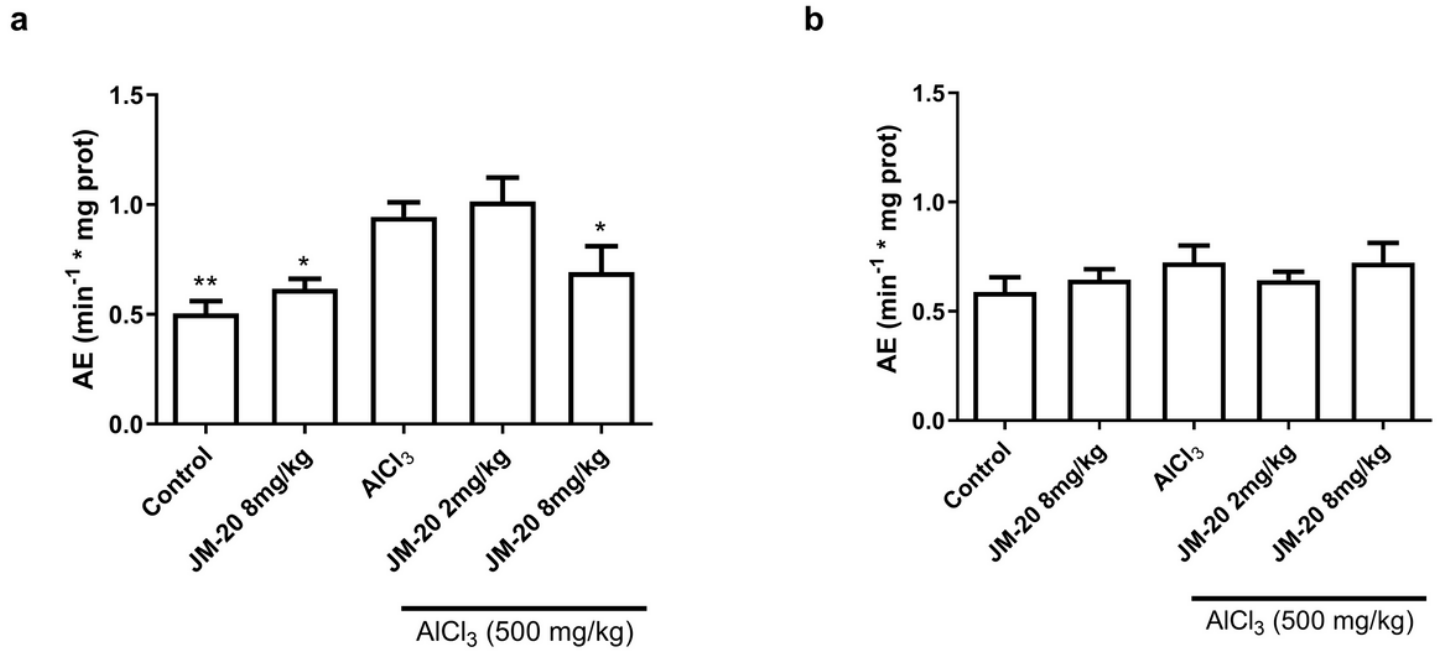

Figure 3

JM-20 prevented the increase in AChE enzymatic activity induced by chronic administration with AlCl3 in (a) the hippocampus and (b) prefrontal cortex of the rat brain. AlCl3 was administered orally for 30 days and during behavioral tests, while JM-20 was co-administered after 15 days after start AlCl3 administration. Bars represent mean ± SEM (n = 9 animals per group). * P <0.05, ** P <0.01, *** P <0.001 versus AlCl3 group by ANOVA and post hoc Tukey Multiple Comparison Test.

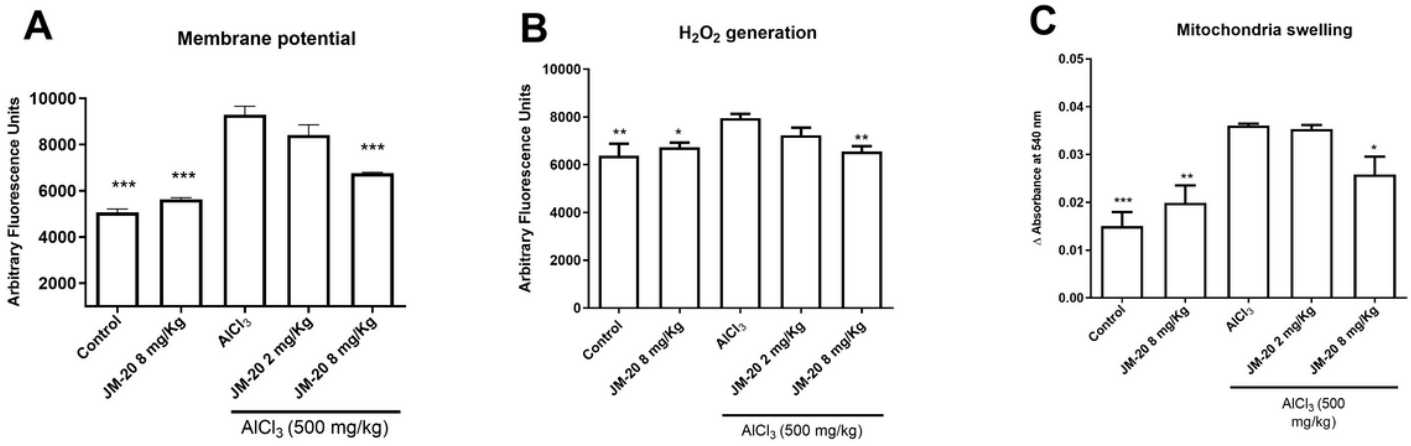

Figure 4

JM-20 reverses the AlCl3-induced significant mitochondrial alterations. Brain mitochondria were isolated after the last behavioral tests, and the membrane potential (a), H2O2 generation (b) and mitochondrial swelling were evaluated. AlCl3 was administered orally for 30 days and during behavioral tests, while JM-

20 was co-administered after 15 days of AlCl3 beginning. Bars represent mean ± SEM (n = 5 per group); * P <0.05, ** P <0.01 **, P <0.001 versus AlCl3 damage group by ANOVA and post hoc Tukey Multiple Comparison Test.

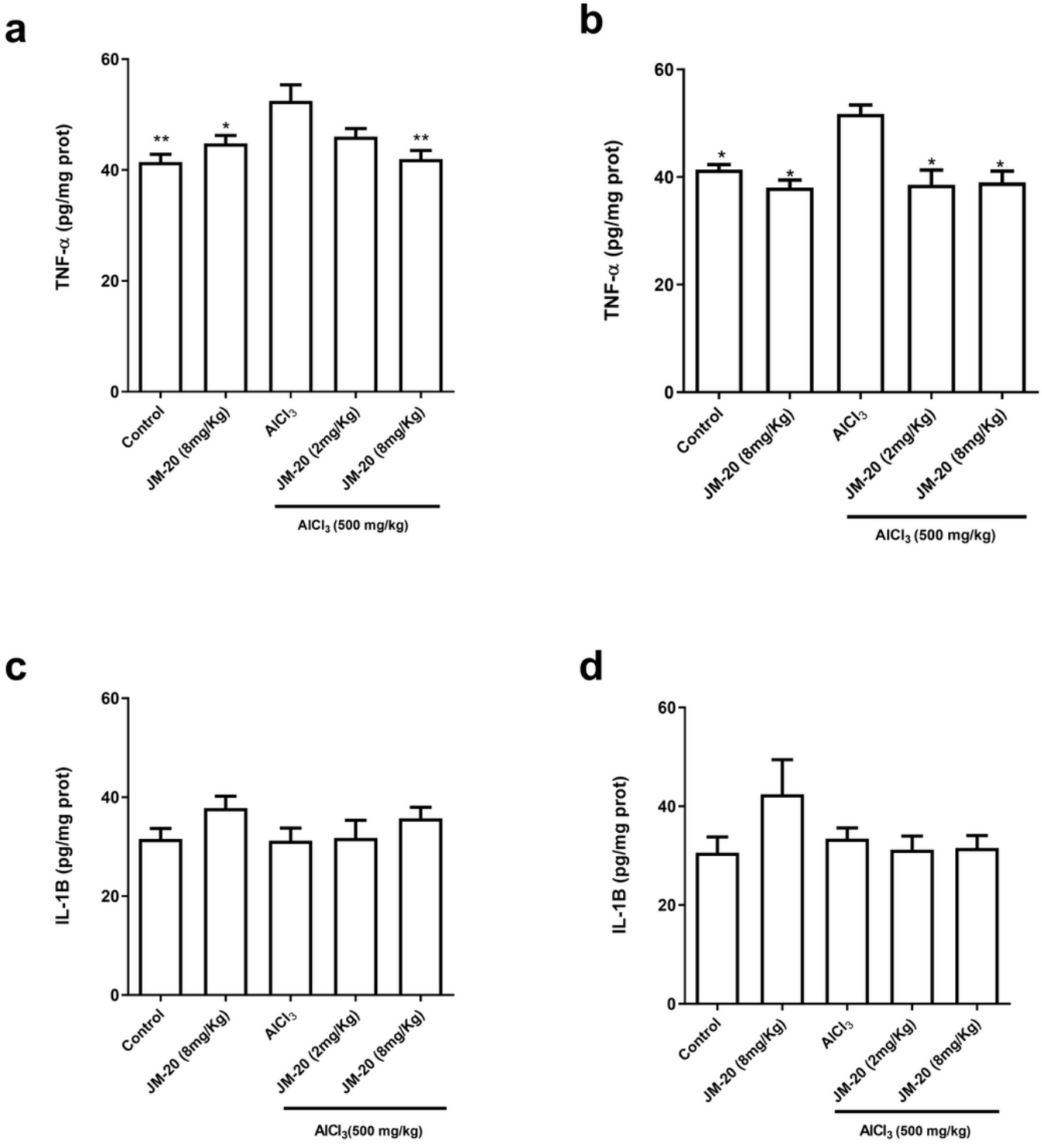

Figure 5

JM-20 reduces hippocampal and prefrontal cortex levels of TNF-α induced by chronic AlCl3 administration, but Il-1β was no significant affected. TNF-α level on hippocampus (a) and prefrontal

cortex (b). Il-1β levels on hippocampus (c) and prefrontal cortex (d). AlCl3 was administered orally for 30 days and during behavioral tests, while JM-20 was co-administered after 15 days of staring AlCl3 administration beginning. Bars represent mean ± SEM (n = 5 per group); * P <0.05, ** P <0.01 **, P <0.001 versus AlCl3 group by ANOVA and post hoc Tukey Multiple Comparison Test.

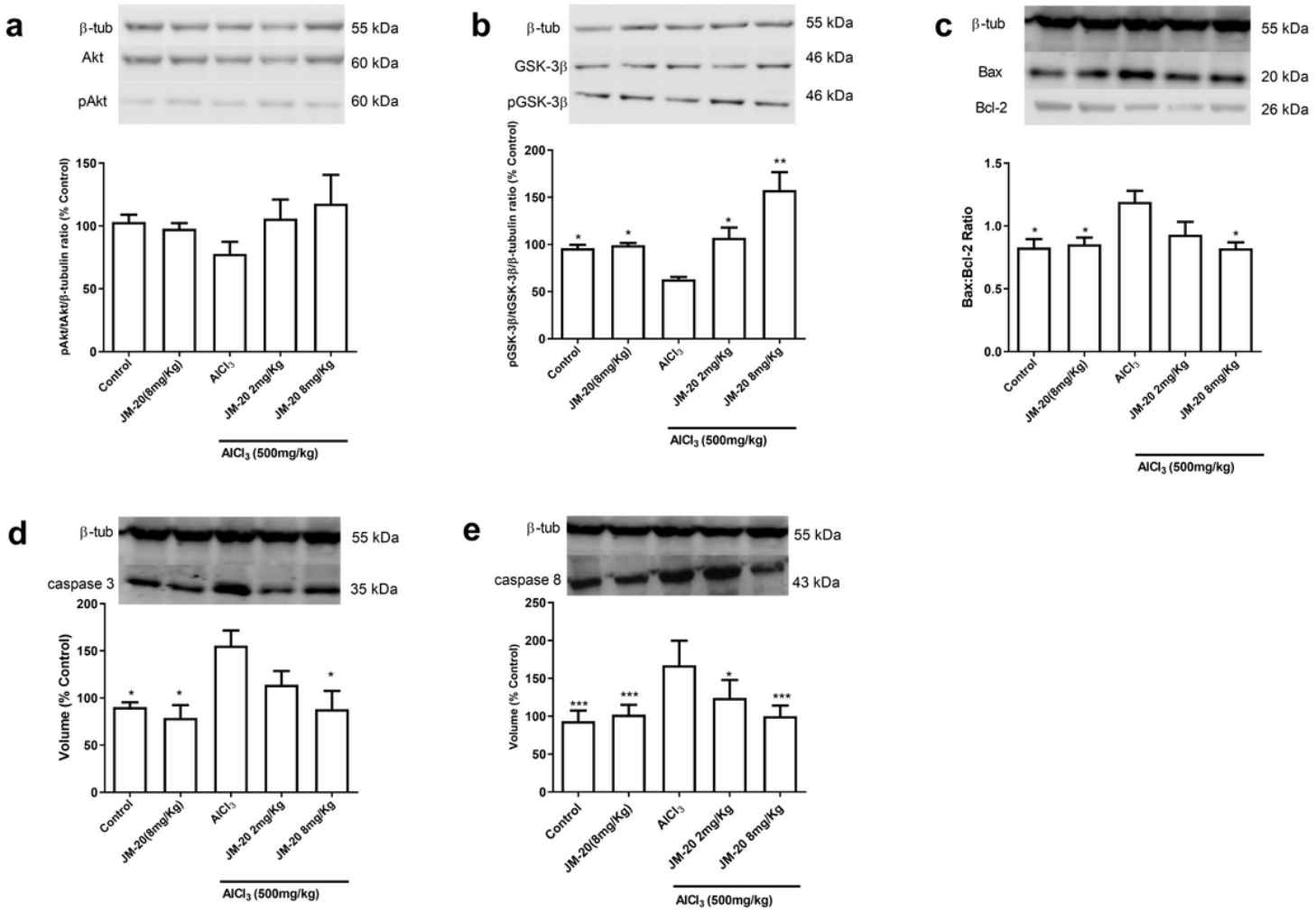

Figure 6

JM-20 increase hippocampal phosphorylation (inactivation) of GSK-3β, maintain normal levels of Bcl-2 over Bax expression, and prevented caspase 3 and caspase 8 over expressions in AlCl3-induced apoptosis markers on rats' brain homogenates. Panels A to E represent data from hippocampus homogenates. (a) Akt/pAkt; (b) GSK-3β /pGSK-3β; (c) Ratio Bax/Bcl-2; (d) caspase 3 and (e) caspase 8. Values represent mean ± SEM (n = 7). * P <0.05, ** P <0.01 **, P <0.001 versus AlCl3 group by ANOVA and post hoc Tukey Multiple Comparison Test.

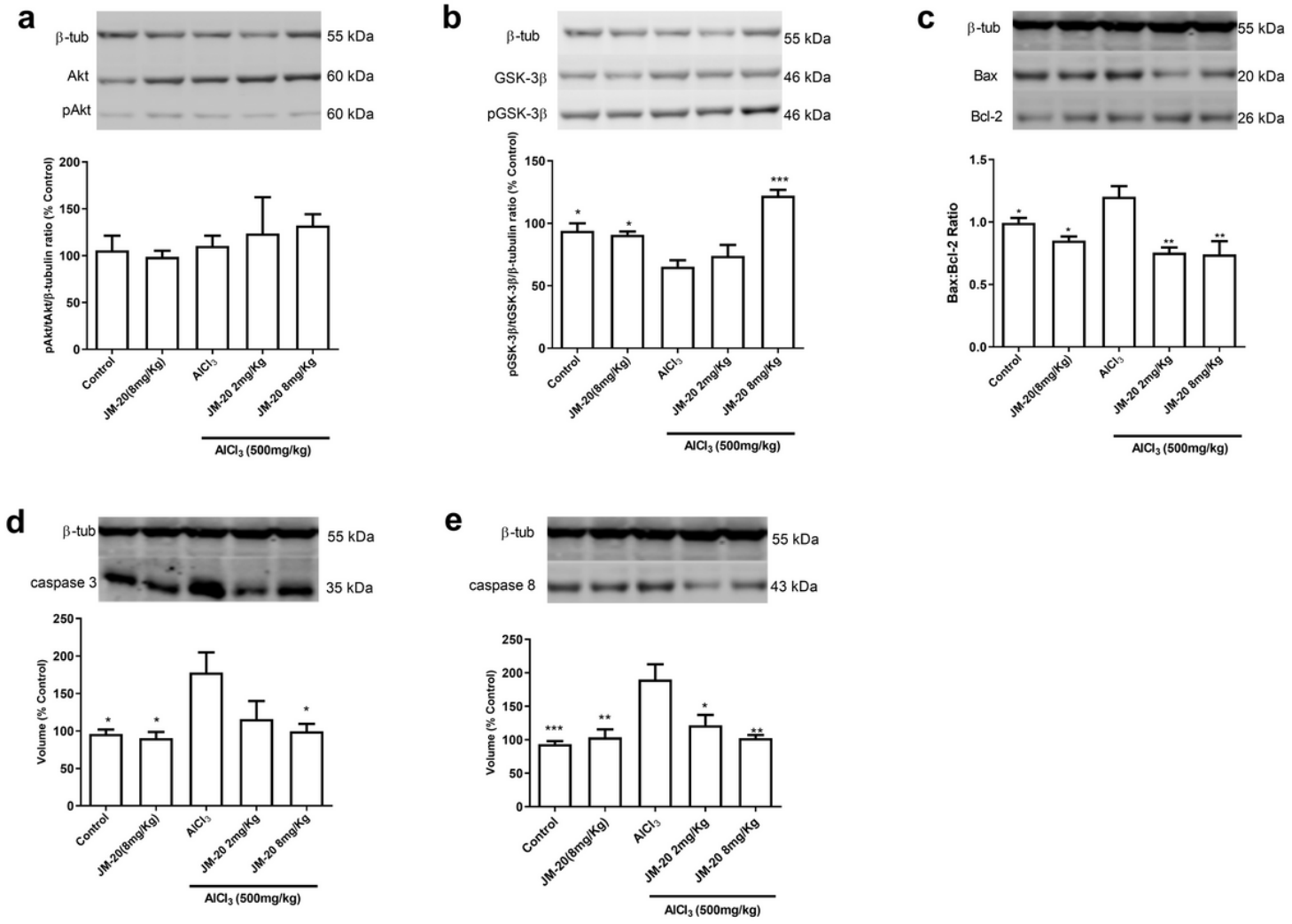

Figure 7

JM-20 increase prefrontal cortex phosphorylation (inactivation) of GSK-3β, maintain normal levels of Bcl-2 over Bax expression, and prevented caspase 3 and caspase 8 over expressions in AlCl3-induced apoptosis markers on rats' brain homogenates. Panels A to E represent data from prefrontal cortex homogenates. (a) Akt/pAkt; (b) GSK-3β /pGSK-3β; (c) Ratio Bax/Bcl-2; (d) caspase 3 and (e) caspase 8. Values represent mean ± SEM (n = 7). * P <0.05, ** P <0.01 **, P <0.001 versus AlCl3 group by ANOVA and post hoc Tukey Multiple Comparison Test.

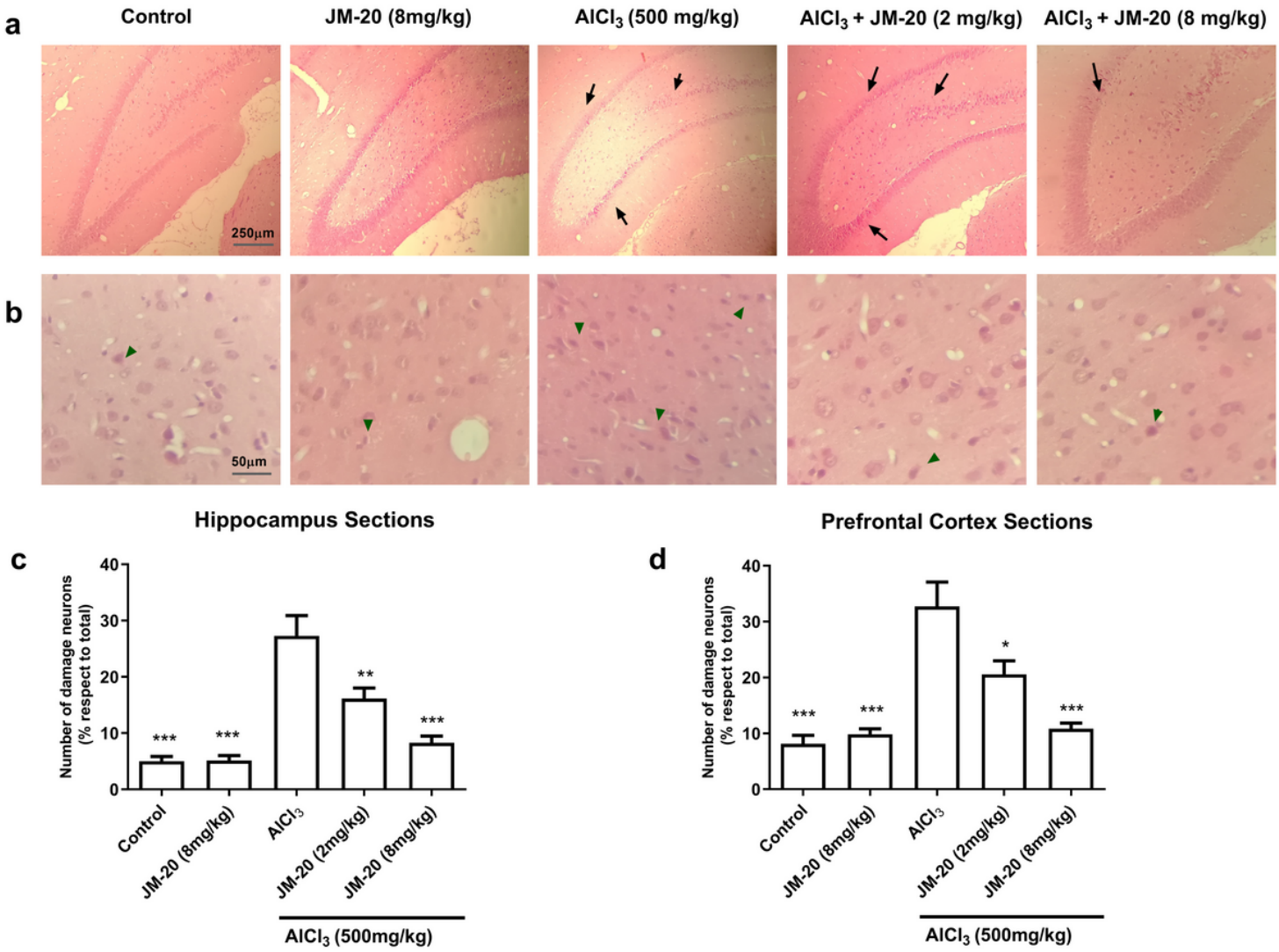

Figure 8

Histological representation of protective effects of JM-20 against AlCl3-induced tissue damage on hippocampus and prefrontal cortex of rat´s brain. The panels show representative micrographs and morphometric analysis in HO (a, c) and PC (b, d). The bars represent the mean ± SEM (n = 5), and ANOVA and post hoc of Tukey was performed. * P < 0,05, ** P <0,01 **, P <0,001 respect to AlCl3 group.