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A multifunctional compound ebselen reverses memory impairment, apoptosis and oxidative stress in a mouse model of sporadic Alzheimer's disease

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ABSTRACT

Alzheimer 's disease (AD) is characterized by progressive cognitive decline including memory impairment, cortical dysfunction, and neuropsychiatric disturbances. The drug discovery to treat AD consists to develop compounds able to act in multiple molecular targets involved in the pathogenesis of the disease and the repositioning of old drugs for new application. This way, the intracerebroventricular (icv) injection of streptozotocin (STZ) has been used as a metabolic model of sporadic AD. The aim of the present study was to investigate whether ebselen (1–10 mg/kg), a multifunctional selenoorganic compound, ameliorates memory impairment, hippocampal oxidative stress, apoptosis and cell proliferation in a mouse model of sporadic AD induced by icv STZ (3 mg/kg, 1 µl/min). The administration of ebselen (10 mg/kg, i.p.) reversed memory impairment and hippocampal oxidative stress, by increasing the activities of antioxidant enzymes and the level of a non-enzymatic antioxidant defense, in Swiss mice administered with icv STZ. The anti-apoptotic property of ebselen was demonstrated by its effectiveness against the increase in the ratios of Bax/Bcl-2, cleaved PARP/PARP and the cleaved caspase-3 levels in the hippocampus of icv STZ mice. Although ebselen reversed memory impairment, it was ineffective against the reduction in the number of BrdU positive cells induced by icv STZ. In conclusion, the multifunctional selenoorganic compound ebselen was effective to reverse memory impairment, hippocampal oxidative stress and apoptosis in a mouse model of sporadic AD induced by icv STZ.

1. Introduction

Alzheimer's disease (AD), the most common form of dementia, is characterized by progressive cognitive decline including memory impairment, cortical dysfunction, and neuropsychiatric disturbances (Paulsen, 2011; Schoonheim et al., 2012). To date, an estimated 35 million people are suffering from AD, and this number is projected to grow to 106.8 million people by 2050 (Sosa-Ortiz et al., 2012). Apart from the slow and progressive degeneration process, the neuropathologic hallmarks of AD are senile plaques and neurofibrillary tangles, along with neuronal and synaptic loss, astrocytic and microglial changes (Selkoe and Hardy, 2016; Walsh and Selkoe, 2004). The development of an appropriate animal model of AD has been difficult, as the etiology of this neurodegenerative disorder is complex and multifactorial (Jack et al., 2018). This way, the intracerebroventricular (icv) injection of streptozotocin (STZ) has been used as a metabolic model of sporadic AD (Grieb, 2016). This model produces central insulin resistance, glucose hypometabolism (Shoham et al., 2003), oxidative stress (Ishrat et al., 2006), inflammation (Shoham et al., 2003), neurodegeneration, and memory impairment (Santos et al., 2012).

Regarding AD therapy, over the last decade, more than 50 candidates have successfully passed phase II clinical trials, but none has passed phase III. Therefore, some major trends in AD drug discovery would be the development of compounds acting on the main stages of the pathogenesis of the disease; the design of drugs acting on multiple molecular targets involved in the pathogenesis of the disease and the

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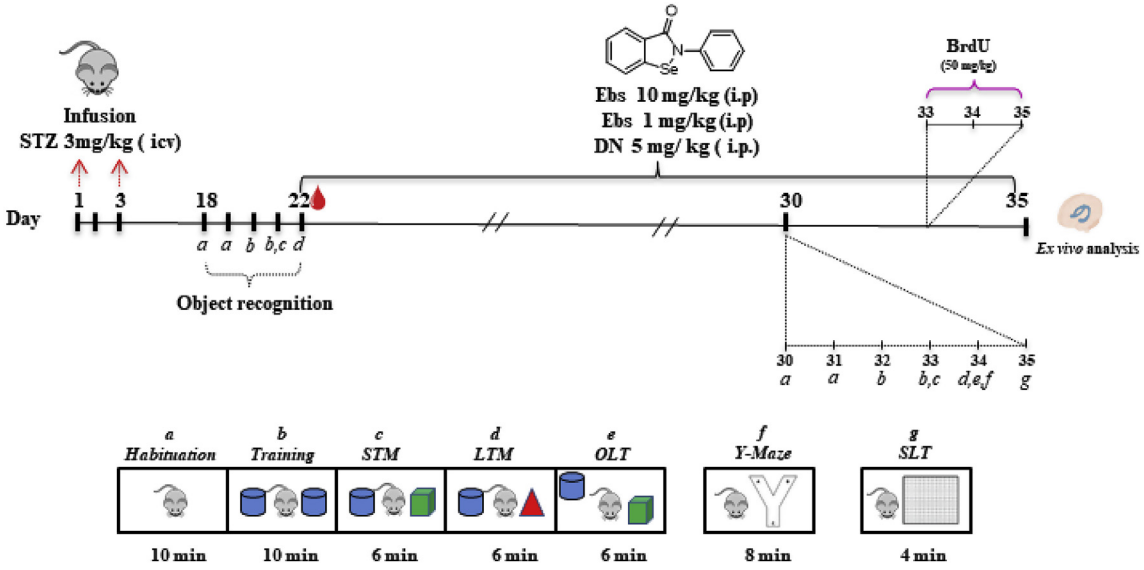

Fig. 1. Schematic representation of the experimental design of this study. Ebs - ebselen, DN - donepezil, STZ - streptozotocin, BrdU- bromodeoxyuridine, STM - short-term memory, LTM-long-term memory, OLT - object location test and SLT - spontaneous locomotor test.

repositioning of old drugs for new (anti-Alzheimer's) application (Bachurin et al., 2017). Ebselen fulfills all these trends because it is a multifunctional selenoorganic compound (Nogueira et al., 2004) currently undergoing clinical trials for cerebral ischemia (Gabryel and Malecki, 2006), bipolar disorder (Masaki et al., 2016; Singh et al., 2013), and noise-induced hearing loss (Mahadevan et al., 2013; Wang et al., 2014). Moreover, the effectiveness of ebselen has been already proven in a transgenic model (Xie et al., 2017), a model of familial AD. Therefore, the aim of the present study was to investigate whether ebselen ameliorates memory impairment, oxidative stress, apoptosis and cell proliferation in a metabolic model of sporadic AD induced by icv STZ.

2. Experimental procedures

2.1. Animals

The experiments were carried out using male adult Swiss mice (25–35 g) obtained from our breeding colony. The mice were housed in cages (5 mice per cage), with free access to food and water. The animals were kept in an air-conditioned room (22 ± 2 °C) under a 12:12 h light/dark cycle, with lights turned on at 7:00 a.m. The experimental procedures of this study were approved by the Institutional Ethics Committee on Care and Use of Experimental Animal Resources from the Federal University of Santa Maria-RS - Brazil (#7372110915). All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments.

2.2. Chemicals

Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one) was prepared and characterized in our laboratory by the method previously described by Engman and Hallberg (1989). Analyses of the ^{1}H NMR and ^{13}C NMR spectra showed analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of ebselen (99.9%) was determined by gas chromatography-mass spectrometry (GC/MS). Streptozotocin (STZ) and 5-bromo-deoxyuridine (BrdU) were obtained from Sigma (St. Louis, MO, USA). Donepezil was purchased from a local pharmacy (Santa Maria, Brazil). All other chemical reagents utilized were obtained from standard commercial suppliers. Ebselen was dissolved in 1: 4: 5 of dimethyl sulfoxide (DMSO), polyethyleneglycol and distilled water. Donepezil and BrdU were dissolved in saline solution. All drugs were administered to mice in a volume of 10 ml/kg. Appropriate vehicle-treated groups were also assessed simultaneously.

2.3. Intracerebroventricular (icv) injection of STZ

Adult male Swiss mice were anaesthetized by the intraperitoneal (ip) route with ketamine at a dose of 100 mg/kg and xylazine at a dose of 5 mg/kg for icv injections. The head of mouse was placed in the stereotaxic apparatus and a midline sagittal incision was made in the scalp. The stereotaxic coordinates for the lateral ventricle were measured accurately as anterio-posterior −0.8 mm, lateral 1.5 mm and dorso-ventral, −4.0 mm relative to bregma and ventral from dura with the tooth bar set at 0 mm. Through a skull hole, a 28-gauge Hamilton® syringe of 10 µl attached to a stereotaxic apparatus and piston of the syringe was lowered manually into lateral ventricle (Kroon and Riley, 1986). STZ (3 mg/kg, icv, 1 µl/min) was dissolved in citrate buffer (3 mg/ml, pH 4.4) (Tiwari et al., 2009) just prior to administration. STZ was injected, on alternate days (days 1 and 3), using a Hamilton microsyringe in a volume of 5 µl/injection in the lateral cerebral ventricle of mice (Deshmukh et al., 2009; Sharma et al., 2010).

After surgery, the animals took approximately 1–2 h to recover from anesthesia. The mice were kept in a well-ventilated room at 25 ± 2 °C in individual cages and provided with free access to food and water until they regained full consciousness. Food and water were placed inside the cage for 2–3 days so that the animals could easily access it without any physical trauma due to head injury.

2.4. Experimental protocol

The experimental design of this study is depicted in Fig. 1. Firstly, in order to validate memory impairment induced by icv STZ, mice performed the object recognition test on days 18–22. At the 22 day of protocol experimental, mice received ebselen at the dose of 1 or 10 mg/kg (ip), or positive control, donepezil, at the dose of 5 mg/kg (ip), once a day for 14 days (35 day) (Blokland, 1995). Regarding the doses of ebselen used in this experimental protocol, the highest dose was selected based on a previously published study (Unsal et al., 2016), which revealed 10 mg/kg (ip) as an effective dose against icv STZ induced oxidative stress and apoptosis in Sprague-Dawley rats. The dose of 1 mg/kg ebselen was carried out in order to investigate whether a lower dose would be effective in this experimental protocol.

The animals (n = 72) were randomly assigned in eight different

groups (n = 9/group) as following: ***Group I***- Sham: mice sham-operated injected with vehicle of STZ and treated with vehicle of ebselen; ***Groups II*** and ***III*** - Sham + ebselen: mice sham-operated injected with vehicle of STZ and treated with ebselen at a dose of 1 or 10 mg/kg, respectively. ***Group IV*** -Sham + donepezil: mice sham-operated injected with vehicle of STZ and treated with donepezil at a dose of 5 mg/kg. ***Group V***- STZ; mice injected with STZ (3 mg/kg, icv) and treated with vehicle of ebselen; ***Groups VI*** and ***VII*** - STZ + ebselen: mice injected with STZ and treated with ebselen at a dose of 1 or 10 mg/kg; ***Group VIII***- STZ + donepezil: mice injected with STZ and treated with donepezil at a dose of 5 mg/kg.

At day 22, which corresponds to the end of validation of memory impairment model, glycemia of animals was measured to rule out the effect of hyperglycemia on behavioral tests and *ex vivo* analyses.

At the end of behavioral tests, mice were immediately killed by cervical dislocation and samples of hippocampus were excised and stored at −80 °C for *ex vivo* analyses (Fig. 1).

2.5. BrdU protocol

A new set of animals, that did not perform the behavioral tests, was used (n = 40) to carry out this experiment. The protocol was performed according to Kim and Sung (2017) and Kee et al. (2002), with some modifications. BrdU was given to mice at a dose of 50 mg/kg once daily for three consecutive days (days 33–35). The animals were euthanized 24 h after the last BrdU injection (Fig. 1).

2.6. Behavioral tests

On days 30–35, the animals performed the behavioral tests as shown in Fig. 1: days 30 and 31- habituation to object recognition test (ORT) arena; days 32 and 33- training of test, day 33 - mice performed training and after that they were subjected to short-term memory test (STM); day 34 - mice performed LTM test (long-term memory), object location test (OLT) and Y-maze test; day 35- mice performed spontaneous locomotor test (SLT) and after that the animals were euthanized.

2.6.1. Object recognition test (ORT)

The object recognition test was performed according to Rosa et al. (2003) with some modifications. The behavioral test was performed in a 45 × 45 cm open field surrounded by 30 cm height walls, made of brown plywood. All animals were given a habituation session where they were left to freely exploring the open field for 10 min. No object was placed in the box during the habituation trial. Subsequently, four objects were used: A1, A2, B and C. The “A” objects were two identical double Lego colorful toys; the “B” object was a double Lego colorful toy “C” object was a double Lego colorful toy. All objects were made of plastic material, with 10 cm × 10 cm (length × height) and presented similar textures, colors (blue, red and yellow), and sizes, but distinctive shapes.

Twenty-four and 48 h after habituation two trainings were carried out by placing each individual mouse for 10 min into the field, in which two identical objects (objects A1 and A2) were positioned in two adjacent corners, 10 cm from the walls. In a STM test given 1.5 h after the last training, the mice explored the open field for 6 min in the presence of one familiar (A) and one novel (B) object. The percentage of the total exploration time that the animal spent investigating the novel object was the measure of recognition memory. Between trials the objects were washed with 10% ethanol solution. In a LTM test given 24 h after the last training, the same mice explored the field for 6 min in the presence of a familiar object A and a novel object C. Recognition memory was evaluated as for the STM test. Exploration was defined as sniffing or touching the object with the nose and/or forepaws. Exploratory preference in: training = (A2/(A1 + A2)) x 100; STM = (B/(A1 + B)) x 100; LTM = (C/(A1 + C)) x 100.

2.6.2. Object location test (OLT)

This test was performed according to Dao et al. (2013) with some modifications, in the same apparatus used in the ORT. The training was carried out by placing each mouse into the field where two objects (objects A1 and C; double Lego colorful toy) were positioned in two adjacent corners, 10 cm from the walls. Thus, 2 h after the session training, object C was moved to a location that was diagonally opposite to object A1, and the animals were allowed to explore the objects during 6 min and the evaluation of the exploration was done as described the steps above in section 2.6.1.

2.6.3. Y- maze test

The Y- maze has three-arm maze with equal angles between all arms, which were 30 cm long and 5 cm wide with 12 cm high walls. The maze floor and walls were constructed from dark grey, polyvinyl plastic. Mice were initially placed within one arm, and the sequence and number of arm entries were recorded manually for each mouse over an 8-min period. The percentage of trials in which the mice entered all three arms (ABC, CAB, or BCA but not ABB) was recorded as an alternation to estimate short-term memory. The Y- maze arms were cleaned with diluted 10% ethanol between tests to remove odors and residues. The alternation score (%) for each mouse was defined as the ratio of the actual number of alternations to the possible number (defined as the total number of arm entries minus two) multiplied by 100 as shown by the following equation: % Alternation = [(Number of alternations)/(Total arm entries − 2)] × 100. The number of arm entries per trial was used as an indicator of locomotor activity (Gotz and Ittner, 2008).

2.6.4. Spontaneous locomotor test (SLT)

With the purpose of excluding sedative or motor abnormality, the mouse performed spontaneous locomotor test. The animals were exposed to the chamber and activity was monitored under light and sound-attenuated conditions. Testing took place in a clear acrylic chamber (500 × 480 × 500 mm) equipped with 16 infrared sensors for the automatic recording of horizontal activity (Model EP149, Insight Instruments Ltda, Sao Paulo, BR). Each animal initially was placed in the center of the testing chamber and allowed to freely move while being tracked by an automated tracking system. The data (distance, speed and crossings) were collected and recorded during 4 min.

2.7. Ex vivo analyses

2.7.1. Glycemia

The glucose levels were determined in portable ACCU-CHEK ACTIVE glucose. The glucose level was expressed as mg dl^{-1}.

2.7.2. Parameters of oxidative stress

The hippocampus samples of mice from all experimental groups were homogenized in 50 mM Tris HCl at pH 7.4, 1:10 (w/v), and centrifuged at 2500 g for 10 min at 4 °C to yield a low-speed supernatant (S1) fraction. S1 was used for the determination of oxidative stress parameters.

2.7.2.1. Malondialdehyde levels (MDA). Briefly, an aliquot of S1 was added to NaOH 3 M and incubated at 60 °C during 30 min. After, 6% H_3PO_4 and 0.8% thiobarbituric acid (TBA) were added to the system and the mixture was heated at 90 °C for 2 h. Following, 10% SDS and *n*-butanol were added to extract the TBA-malondialdehyde (MDA) product, which was analyzed on Shimadzu® HPLC equipment. The analytical column was a Phenomenex® ODS-2C_{18} reverse-phase (250 mm × 4.6 mm, 5 µm; 100 Å, Allcrom, BR) and the mobile phase was ultrapure water and methanol (50:50; v/v). The HPLC analysis was performed under isocratic conditions at a 0.6 ml/min flow rate and UV detector set at 532 nm with a 20 µl sample volume injection (Grotto et al., 2007). The results were expressed as nmol MDA/mg protein.

2.7.2.2. Catalase (CAT) activity. The CAT activity was spectrophotometrically assayed by monitoring the H_2O_2 consumption at 240 nm according to Aebi (1984). The enzymatic reaction was performed by the addition of an S1 aliquot and the substrate (H_2O_2) at a concentration of 0.3 mM in a medium containing 50 mM phosphate buffer, pH 7.0. The enzymatic activity was expressed in units (1 U decomposes 1 μmol of H_2O_2/min at pH 7.0 and at 25 °C)/mg protein.

2.7.2.3. Superoxide dismutase (SOD) activity. The SOD activity was measured spectrophotometrically according toMisra and Fridovich (1972). This method is based on the capacity of SOD in inhibiting autoxidation of epinephrine to epinechrome. In this assay, S1 diluted 1:10 (v/v) was added in a 50 mmol/L Na_2CO_3 buffer pH 10.3 and the enzymatic reaction was initiated by adding epinephrine. The color reaction was measured at 480 nm and the results were express in units (1 U decomposes 1 μmol of epinephrine/min at pH 7 and at 25 °C)/mg protein.

2.7.2.4. Glutathione S-transferase (GST) activity. The GST activity was assayed spectrophotometrically at 340 nm by the method ofHabig et al. (1974). The reaction mixture contained an aliquot of S_1, 0.1 M potassium phosphate buffer pH 7.4, 100 mM glutathione (GSH) and 100mM1-Chloro-2,4-dinitrobenzene (CDNB), which was used as substrate. The enzymatic activity was expressed as nmol CDNB conjugated min/mg/protein.

2.7.2.5. Total non-protein sulfhydryl (NPSH) levels. The NPSH levels were determined by the method of Ellman et al. (1961). S_1 was mixed (1:1) with 10% trichloroacetic acid. After the centrifugation (2500 g for 10 min), the protein pellet was discarded and free – SH groups were determined in the clear supernatant. An aliquot of supernatant was added in 1M potassium phosphate buffer (pH 7.4) and 10 mM 5.5′-dithiobis – (2 – nitrobenzoic acid). The color reaction was measured at 412 nm. NPSH levels were expressed as nmol NPSH/g tissue.

2.7.2.6. Protein quantification. The protein concentration was measured by the method described byBradford (1976), using bovine serum albumin (1 mg/ml) as the standard.

2.7.3. BrdU immunohistochemistry

The animals (n = 6–9) were deeply anaesthetized and perfused with saline followed by cold 4% paraformaldehyde (PFA). Brains were removed, fixed in 4% PFA and embedded in paraffin. For BrdU immunohistochemistry, 5 μm brain sections were cut through the hippocampus, and slices were first deparaffinized (40 min at 80 °C) and rinsed in xylol. Sections were then rinsed in phosphate-buffered saline (PBS, pH 7.00) and the blockade of non-specific proteins were made with 1% of bovine serum albumin (BSA) diluted in PBS. Afterward, the blockade of endogenous peroxidase were made with 5% H_2O_2 in methanol (3 times) and washed in 0.05% Triton X-100 diluted in PBS, and then were incubated with a mouse monoclonal anti-BrdU antibody (RPN 202 Kit, GR Healthcare®, 1:100) diluted in DNAse-1 overnight at 4 °C. Subsequently, the sections were incubated with a secondary antibody peroxidase anti-mouse-IgG, followed by tertiary antibody (both Spring®) 40 min each, at room temperature. The immunohistochemical reaction was revealed by 0.06% 3,3-diaminobenzidine (DAB) in PBS for 5 min. After being rinsed in distilled water, sections were counterstained with hematoxylin for 5 s, dehydrated in ethanol and mounted on slides using Entellan. For each brain, BrdU-positive cells were identified by their brown stain and were counted visually using a *Leica Application Suite X* microscope at 40× objective magnification. The number of stained BrdU cells was determined through mean of 10 cuts per animal with an interval of 50 μm in the dentate gyrus area of hippocampus. Data were expressed as total number of BrdU positive cells in the dentate gyrus.

2.7.4. Western blot assay

For the western blot analyses, the contents of proteins were not determined in the samples from mice of icv STZ treated with ebselen (1 mg/kg) and its respective control group because this dose of ebselen was not effective in all behavioral tests.

Samples of hippocampus (n = 5–6 animals/group) were homogenized in Radioimmunoprecipitation assay buffer (RIPA buffer) solution containing 150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, in the presence of commercial phosphates and protein inhibitor cocktail (Sigma-Aldrich Company, St. Louis, Missouri, United States). Tissue extracts were diluted to a final protein concentration of 2 μg/μl. The samples (40 μg of protein) and pre stained molecular weight standards (Sigma-Aldrich Company, St. Louis, Missouri, United States) were separated on 10% resolving with 4% concentrating SDS-PAGE electrophoresis gels. Proteins were transferred to nitrocellulose membrane using Transfer-Blot® Turbo™ Transfer System (1.0 A; 45 min for proteins above 25 kDa or 5 min for proteins below 25 kDa). After blocking with 3% bovine serum albumin solution, the blots were incubated overnight at 4 °C with rabbit anti-poly (ADP-ribose) polymerase (PARP) (1:1000); rabbit cleaved caspase- 3 (caspase- 3) (1:1000); and rabbit (Bcl - 2) (1:1000) obtained from Cell Signaling Technology, Beverly, MA, USA and rabbit (Bax) (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, US). Mouse anti-β-actin (1:5000, abcam) was stained as additional control of the protein loading. After primary antibodies incubation, membranes were washed and incubated with secondary antibodies conjugated with horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA, USA) for 1 h at room temperature and developed with chemiluminescence kit (Amersham, São Paulo/Brazil). Optical density (OD) of the Western blotting bands was quantified using Image J (NIH, Bethesda, MD, USA) software for Windows. Each value was derived from the ratio between arbitrary units obtained by the protein band and the respective β-actin band.

2.8. Statistical analysis

All experimental results are presented as the mean ± S.E.M. Normality of data was analyzed using a D'Agostino and Pearson omnibus normality test. Student *t*-test was used to compare STZ and vehicle groups in the object recognition test (in the validation model of memory impairment). Statistical comparisons among experimental groups (STZ x ebselen) and (STZ x donepezil) in behavioral tests and parameters of oxidative stress were performed by two-way analysis of variance followed by the Newman–Keuls test when appropriate. Immunohistochemistry and western blot data were statistically compared by one-way analysis of variance followed by the Newman–Keuls test when appropriate. All analyses were performed by using the STATISTICA for Windows software Version 7 (Stat Soft, Oklahoma, USA) by an investigator blinded to treatment. A value of $P < 0.05$ was considered to be significant.

3. Results

3.1. STZ induced memory impairment in mice

Fig. 2 shows the effect of STZ injection on the performance of mice in the object recognition test. The Student's *t*-test revealed that STZ decreased the exploratory preference (% time spent exploring a novel object) of mice when compared with that of the vehicle-treated group in the STM ($P = 0.006$ and LTM ($P = 0.0001$) tests.

By contrast, the insert of Fig. 2 demonstrates that STZ did not alter the time spent exploring any of the two identical objects in training ($P = 0.480$).

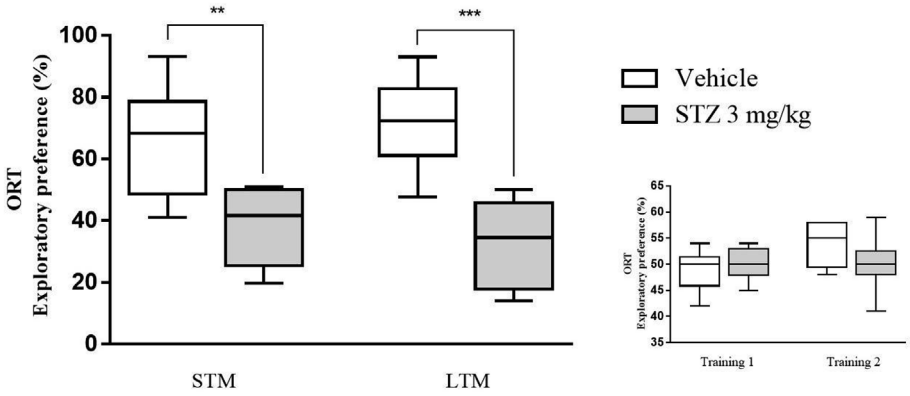

Fig. 2. Effects of STZ on the mouse performance in the ORT. Insert shows the performance of mice in training (percentage of time spent exploring any of two identical objects). Values are expressed as mean ± S.E.M. of 9–10 animals. Asterisks denote the significance levels when compared with the respective vehicle group: (**) $P < 0.01$ and (***) $P < 0.001$ (Student's *t*-test). STM - short-term memory; LTM - long-term memory.

3.2. Ebselen reversed memory impairment induced by STZ in the mouse ORT

Fig. 3 shows the effect of ebselen treatment on the performance of mice in the ORT. There were no significant differences among groups in the time spent exploring any of the two identical objects in trainings 1 and 2 [$F_{(2,48)} = 0.896$, $P = 0.415$](Fig. 3A).

The two-way ANOVA of percentage of exploratory preference revealed a significant ebselen × STZ interaction in the STM [$F_{(2,48)} = 5.38$, $P = 0.007$] and in the LTM [$F_{(2,48)} = 5.64$, $P = 0.006$]. Post-hoc analyses demonstrated that both doses of ebselen reversed memory impairment induced by STZ of mice in the STM (Fig. 3B), but only the highest dose of ebselen increased the percentage of exploratory preference of mice in the LTM (Fig. 3C).

The inserts of figures demonstrate the effects of donepezil, the positive control, in the training session (Fig. 3A), STM (Fig. 3B) and LTM (Fig. 3C). There were no significant differences among groups in the time spent exploring any of the identical objects in trainings 1 and 2 [$F_{(1,32)} = 0.479$, $P = 0.493$]. The two-way ANOVA of percentage of exploratory preference revealed a significant donepezil × STZ interaction in the STM [$F_{(1,32)} = 41.34$, $P = 0.0001$], but not in the LTM [$F_{(1,32)} = 2.61$, $P = 0.115$]. Post hoc analysis showed that donepezil at a dose of 5 mg/kg reversed memory impairment induced by STZ in mice only in the STM (Fig. 3C).

3.3. Ebselen reversed memory impairment induced by STZ in the mouse OLT

Fig. 4 shows the effect of ebselen treatment on the performance of mice in the OLT. The two-way ANOVA of percentage of exploratory preference revealed a significant ebselen × STZ interaction in the OLT [$F_{(2,48)} = 4.44$, $P = 0.016$]. Post hoc comparisons showed that STZ decreased the percentage of exploratory preference of mice in the OLT when compared with that of the vehicle-treated group, and ebselen at a dose of 10 mg/kg reversed this parameter.

The insert of Fig. 4 demonstrates the effect of the positive control, donepezil, in the OLT. The two-way ANOVA of percentage of exploratory preference indicated a significant donepezil × STZ interaction in the OLT [$F_{(1,32)} = 14.04$, $P = 0.0007$]. Post hoc analysis showed that donepezil at a dose of 5 mg/kg reversed the STZ– induced mouse memory impairment in the OLT.

3.4. Ebselen reversed memory impairment induced by STZ in the mouse Y - maze

Fig. 5 shows the effect of ebselen treatment on the performance of mice in the Y- maze test. The two-way ANOVA of alternations (%) indicated a significant ebselen × STZ interaction [$F_{(2,48)} = 3.21$, $P = 0.049$]. Post hoc comparisons showed that STZ decreased the percentage of alternations of mice in the Y-maze test when compared with that of the vehicle-treated group, and ebselen at both doses reversed this parameter.

The insert of Fig. 5 demonstrates the effect of the positive control, donepezil, in the Y - maze test. The two-way ANOVA of alternations (%) demonstrated a significant donepezil × STZ interaction in the Y - maze [$F_{(1,32)} = 11.13$, $P = 0.002$]. Post hoc analysis showed that donepezil at a dose of 5 mg/kg reversed STZ – induced mouse memory impairment in the Y-maze test.

3.5. Ebselen and STZ did not alter the mouse spontaneous locomotor activity

Table 1 shows the effect of ebselen treatment or STZ on the mouse spontaneous locomotor activity. The two-way ANOVA of crossings [$F_{(2,42)} = 0.077$, $P = 0.92$], distance travelled [$F_{(2,42)} = 0.596$, $P = 0.55$] and speed [$F_{(2,42)} = 0.031$, $P = 0.96$] revealed that there was not a statistically significant ebselen × STZ interaction. Neither STZ nor ebselen changed the number of crossings, distance travelled and speed of mice.

The two-way ANOVA of crossings [$F_{(1,28)} = 0.77$, $P = 0.78$], distance travelled [$F_{(1,28)} = 2.01$, $P = 0.16$] and speed [$F_{(1,28)} = 0.055$, $P = 0.81$] did not show a statistically significant donepezil × STZ interaction. The data on spontaneous locomotor activity parameters were similar in all experimental groups (Table 1).

3.6. Ebselen reversed oxidative stress induced by STZ in hippocampus of mice

Fig. 6A–E shows the ebselen effects on parameters of oxidative stress in the mouse hippocampus. The two-way ANOVA of MDA levels in the mouse hippocampus [$F_{(2,42)} = 3.25$, $P = 0.048$] revealed a significant ebselen × STZ interaction. Post hoc analyses showed that STZ increased MDA levels when compared with those of the vehicle-treated group. Treatment of mice with ebselen was effective against this parameter (Fig. 6A) only at the dose of 10 mg/kg.

The two-way ANOVA of CAT activity revealed a significant ebselen × STZ interaction [$F_{(2,36)} = 5.38$, $P = 0.008$]. Post hoc analysis showed that STZ decreased the CAT activity when compared with that of the vehicle-treated group, and ebselen at the highest dose was effective against this parameter (Fig. 6B).

The two-way ANOVA of SOD activity showed a significant ebselen × STZ interaction [$F_{(2,42)} = 13.94$, $P = 0.0001$]. Post hoc analysis showed that STZ decreased the SOD activity when compared with the vehicle-treated group and treatment of mice with ebselen reversed this parameter only at the dose of 1 mg/kg (Fig. 6C).

The two-way ANOVA of GST activity indicated a significant ebselen × STZ interaction [$F_{(2,36)} = 3.58$, $P = 0.037$]. Pos hoc analysis showed that STZ increased the GST activity when compared with the vehicle-treated group and treatment of mice with ebselen was effective against this parameter at both doses tested (Fig. 6D).

The two-way ANOVA of NPSH levels revealed a significant

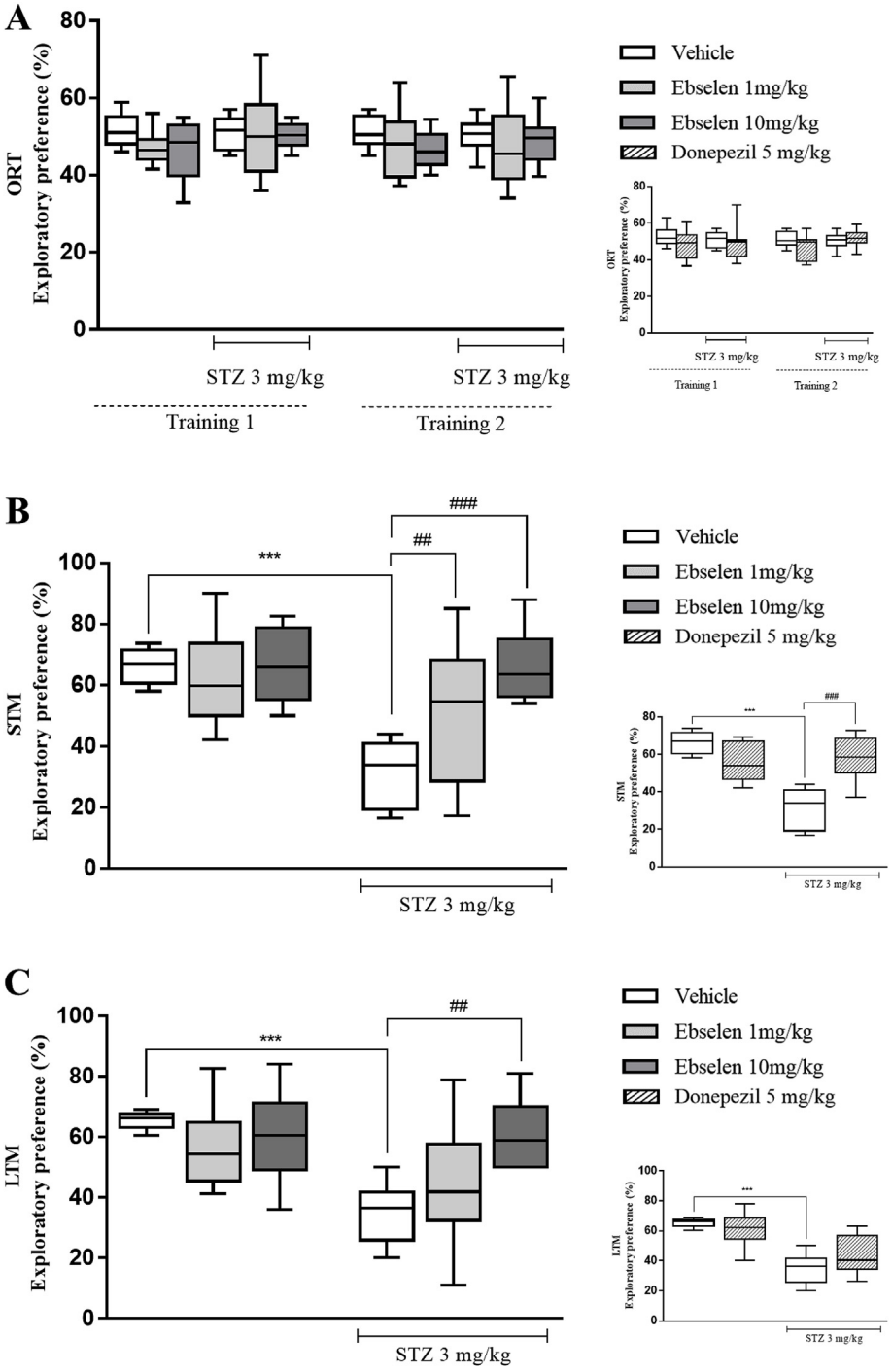

Fig. 3. Effects of ebselen on training sessions (A), STM (B) and LTM (C) in the ORT of mice treated with STZ. Inserts show effects of donepezil treatment (5 mg/kg) on the performance of mice in training sessions (A), STM (B) and LTM (C). Values are expressed as mean ± S.E.M. of 9 animals. Asterisk denotes the significance levels when compared with the respective vehicle group: (***) $P < 0.001$. Hashtag denotes the significance levels when compared with the STZ group: ($^{##}$) $P < 0.01$ and ($^{###}$) $P < 0.001$ (two-way ANOVA followed by the Newman Keuls). STM - short-term memory; LTM - long-term memory.

ebselen × STZ interaction [$F_{(2,30)} = 4.23$, $P = 0.024$]. Pos hoc analysis showed that STZ decreased the NPSH levels when compared with the vehicle-treated group and treatment of mice with ebselen reversed this parameter only at the dose of 10 mg/kg (Fig. 6E).

The inserts of Fig. 6 A-E demonstrate the effect of positive control, donepezil, in the parameters of oxidative stress. The two-way ANOVA of MDA levels indicated a significant donepezil × STZ interaction [$F_{(1,28)} = 4.62$, $P = 0.040$]. Post hoc analysis showed that treatment of mice with donepezil at a dose of 5 mg/kg was effective against the increase in MDA levels (Fig. 6A) induced by STZ in the mouse hippocampus.

The two-way ANOVA of CAT activity did not reveal a significant effect of donepezil [$F_{(1,24)} = 0.378$, $P = 0.544$] (Fig. 6B).

The two-way ANOVA revealed a significant donepezil × STZ interaction in the SOD activity [$F_{(1,24)} = 8.37$, $P = 0.007$]. Post hoc analysis showed that STZ decreased the SOD activity when compared with the vehicle-treated group and treatment of mice with donepezil at a dose of 5 mg/kg reversed this parameter in the hippocampus (Fig. 6C).

The two-way ANOVA revealed a significant donepezil × STZ interaction in the GST activity [$F_{(1,24)} = 8.02$, $P = 0.009$]. Pos hoc analysis

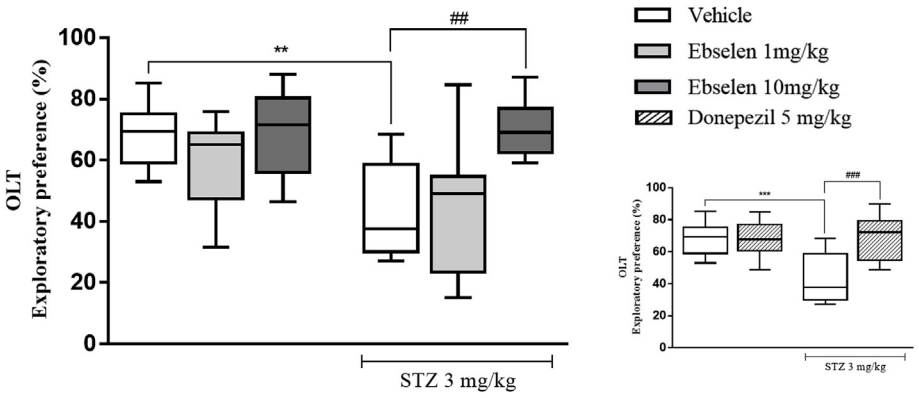

Fig. 4. Effects of ebselen on the OLT of mice treated with STZ. Insert shows the effect of donepezil treatment (5 mg/kg). Values are expressed as mean ± S.E.M. of 9 animals. Asterisk denotes the significance levels when compared with the respective vehicle group: (**) P < 0.01 and (***) P < 0.001. Hashtag denotes the significance levels when compared with the STZ, vehicle treated group ($^{\#\#}$) P < 0.01 and ($^{\#\#\#}$) P < 0.001 (two-way ANOVA followed by the Newman Keuls). LTM - long-term memory.

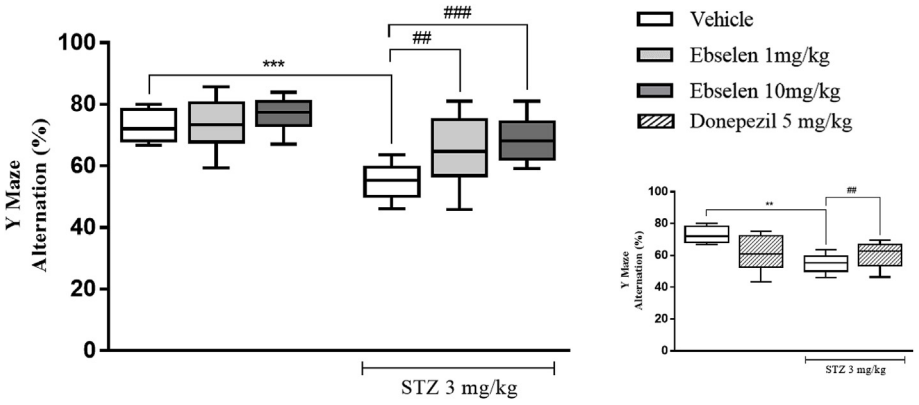

Fig. 5. Effects of ebselen on Y- maze of mice treated with STZ. Insert shows the effect of donepezil treatment (5 mg/kg). Values are expressed as mean ± S.E.M. of 9 animals. Asterisk denotes the significance levels when compared with the respective vehicle group: (**) P < 0.01 and (***) P < 0.001. Hashtag denotes the significance levels when compared with the STZ, vehicle treated group ($^{\#\#}$) P < 0.01 and ($^{\#\#\#}$) P < 0.001 (two-way ANOVA followed by the Newman Keuls).

Table 1
Ebselen and icv STZ effects on the mouse spontaneous locomotor test.

Groups	Number of Crossings	Distance (mm)	Speed (mm/s)
Vehicle	607.0 ± 55.86, *p = 0.775*	10060 ± 803, *p = 0.847*	46.0 ± 2.0, *p = 0.658*
Ebs 1 mg/kg	632.5 ± 34.78, *p = 0.937*	10560 ± 729, *p = 0.849*	52.0 ± 3.0, *p = 0.815*
Ebs 10 mg/kg	552.0 ± 44.38, *p = 0.996*	9605 ± 703,*p = 0.831*	48.0 ± 3.2, *p = 0.838*
DN 5 mg/kg	645.5 ± 49.30, *p = 0.951*	10990 ± 526, *p = 0.460*	45.8 ± 2.2, *p = 0.904*
STZ	530.0 ± 65.14, *p = 0.775*	9428 ± 950, *p = 0.847*	43.3 ± 4.9, *p = 0.658*
STZ + Ebs1 mg/kg	615.0 ± 45.94, *p = 0.981*	11640 ± 525, *p = 0.811*	49.8 ± 1.5, *p = 0.609*
STZ + Ebs10 mg/kg	669.0 ± 53.94, *p = 0.934*	11698 ± 869, *p = 0.931*	44.2 ± 4.5, *p = 0.944*
STZ + DN 5 mg/kg	647.5 ± 51.69, *p = 0.915*	10770 ± 960, *p = 0.597*	46.4 ± 4.7, *p = 0.833*

Values are expressed as mean ± S.E.M. of 8–9 animals. Data analysis was carried out through two-way analysis of variance ANOVA followed by the Newman-Keul's test. P values of *per se* groups (Ebs 1 and 10 mg/kg, DN) were obtained by comparing these groups with the vehicle group, and those of STZ-treated groups (STZ + ebselen (1 and 10) and STZ + DN) were obtained by comparing with the STZ group. Ebs – ebselen; DN – donepezil; STZ – streptozotocin.

showed that STZ increased the GST activity when compared with the vehicle-treated group and treatment of mice with donepezil at a dose of 5 mg/kg reversed this parameter (Fig. 6D).

The two-way ANOVA revealed a significant donepezil × STZ interaction in the NPSH levels [$F_{(1,20)} = 4.36$, P = 0.049]. Post hoc analysis showed that STZ decreased the NPSH levels when compared with the vehicle-treated group and treatment of mice with donepezil at a dose of 5 mg/kg increased this parameter in the hippocampus (Fig. 6E).

3.7. Icv STZ did not alter blood glucose levels

Table 2 shows the effect of icv STZ on glycemia. An unpaired Student's *t*-test revealed that icv STZ did not alter glycemia in mice when compared with that of the vehicle group (P = 0.610).

3.8. Ebselen did not restore reduced cell proliferation induced by STZ in the mouse dentate gyrus of hippocampus

Fig. 7 shows the ebselen effect on the number of BrdU positive cells, a marker of cell proliferation, in the dentate gyrus area of hippocampus. The one-way ANOVA revealed the reduction on the number of BrdU positive cells in the dentate gyrus area of hippocampus of animals injected with STZ when compared with those of the vehicle-treated group [$F_{(4,34)} = 21.69$, P = 0.0001]. By contrast, one-way ANOVA showed that neither ebselen nor donepezil restored cell proliferation in the dentate gyrus area of hippocampus (P = 0.210).

3.9. Ebselen reversed the levels of apoptotic proteins altered by STZ in the hippocampus of mice

The Bax/Bcl-2 and PARP cleaved/PARP ratios; and the protein levels of cleaved caspase- 3 in the hippocampus of mice are shown in Fig. 8A–C. There was a significant increase in the ratio of Bax/Bcl-2 [F

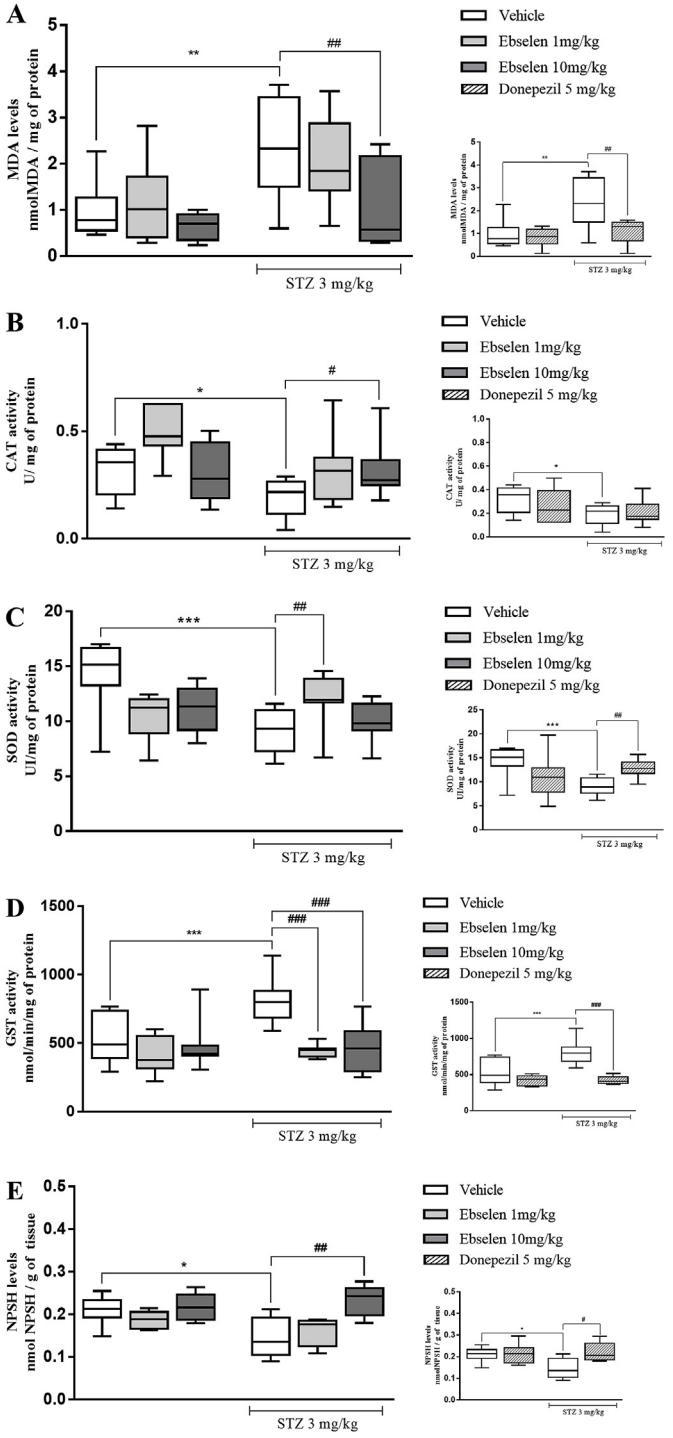

Fig. 6. Effects of ebselen on hippocampal MDA levels (A), CAT activity (B), SOD activity (C), GST activity (D) and NPSH levels (E) of mice treated with STZ. Inserts show the effects of donepezil treatment (5 mg/kg). Values are expressed as mean ± S.E.M. of 8 (MDA), 7 (CAT, SOD and GST) and 6 (NPSH) animals. Asterisk denotes the significance levels when compared with the respective vehicle treated group: (*) $P < 0.05$, (**) $P < 0.01$ and (***) $P < 0.001$. Hashtag denotes the significance levels when compared with the STZ, vehicle treated group ($^{\#}$) $P < 0.05$, ($^{\#\#}$) $P < 0.01$ and ($^{\#\#\#}$) $P < 0.001$ (two-way ANOVA followed by the Newman Keuls).

$_{(5,29)} = 14.59$, $P = 0.0001$] and cleaved PARP/PARP [$F_{(5,29)} = 12.28$, $P = 0.0001$] and in the level of cleaved caspase- 3 [$F_{(5,31)} = 5.16$, $P = 0.002$] in the hippocampus of mice injected with STZ when compared to those of the vehicle-treated group. Ebselen and donepezil reversed this increase to the levels of control group.

The levels of proteins Bax [$F_{(5,30)} = 14.59$, $P = 0.0001$] and Bcl-2 [$F_{(5,30)} = 3.96$, $P = 0.0087$] separately plotted are depicted in Fig. 1S.

4. Discussion

The results of the present study demonstrate that the multifunctional compound ebselen reversed memory impairment and modulated oxidative stress and the levels of proteins related to apoptosis in a mouse model of sporadic AD induced by icv STZ.

Animal models are critical to drug discovery and provide a basic platform to investigate new therapies. The icv injection of STZ is an established, standardized and reproducible approach to metabolic sporadic AD (Salkovic-Petrisic et al., 2013). STZ alters the function of enzymes involved in the brain glucose metabolism (Hoyer and Lannert, 2007), stimulates oxidative stress (Agrawal et al., 2011; Ishrat et al., 2009; Javed et al., 2012), the apoptotic pathway (Agrawal et al., 2011) and the release of cytotoxic factors, which can lead to neuronal death (Bhalala, 2015). Although icv STZ is a non-transgenic metabolic model of sporadic AD, which resembles features in brains of AD patients, we should acknowledge the lack of mechanistic explanation of icv STZ action as a limitation of this model (Grieb, 2016).

In the present study, the icv injection of STZ besides causing memory impairment and oxidative stress, it increased the hippocampal levels apoptotic markers and decreased neurogenesis in the dentate gyrus area of hippocampus. Despite differences between STZ doses, some authors Mishra et al. (2018); Kraska et al. (2012) have reported that icv STZ induced severe atrophy of the area around the lateral ventricles after 21 days and lesions extended posterior to the dentate gyrus, and neurodegenerative lesions associated with inflammation and oxidative stress (Kraska et al., 2012). The STZ model has also behavioral consequences, such as memory and learning impairment (Mehla et al., 2013). In fact, our findings demonstrate that icv STZ decreased mouse performance in the STM, LTM, OLT and Y-maze tests, reflecting loss of memory.

Scientists have used a database of 'failed' drugs, found to be safe but ineffective for their proposed use, to identify ebselen, a compound which displays formidable benefit during the animal model phase of research (Noguchi, 2016; Nosengo, 2016) and have been able to reproduce these effects in human clinical trials, as a possible alternative to treat bipolar disorder (Masaki et al., 2016; Singh et al., 2013), and noise-induced hearing loss (Mahadevan et al., 2013; Wang et al., 2014). In fact, ebselen neuroprotective effects have been recognized (Singh et al., 2016) and its multifactorial targets (Luo et al., 2013; Parnham and Sies, 2013) seem to be an advantage for prospective therapeutic strategies. Besides, the effectiveness of ebselen has been already proven in a transgenic model of AD (Xie et al., 2017). Widely used transgenic mouse AD models have provided valuable insights into the molecular mechanisms underlying the memory decline; however, due to the particular β -amyloid-related gene manipulation, they resemble the familial but not the sporadic AD form. Despite the differences in the experimental protocol (dose, species, time of administration and route) between the Xie et al. (2017) study and our present study, the demonstration that ebselen improved the cognitive impairment in spatial learning, reduced β-amyloid level and inhibited tau hyperphosphorylation in a model of familial AD motivated us to investigate this compound in a model that mimics a specific endophenotype, such as STZ icv-treated animals which develop insulin resistant brain state.

The findings of the present study reproduced well-known properties of ebselen, such as antioxidant, anti-apoptotic, neuroprotective and memory enhancing (Singh et al., 2016) in a mouse model of sporadic AD induced by icv STZ. Ebselen administration reversed hippocampal oxidative stress induced by icv STZ by increasing the activities of antioxidant enzymes and the levels of a non-enzymatic antioxidant defense. The anti-apoptotic property of ebselen was demonstrated in this study by its effectiveness against the increase in the ratios of Bax/Bcl-2,

Table 2
Effect of icv STZ on glycemia of mice.

Groups	Glycemia (mg/dl)
Vehicle	107.7 ± 4.86
STZ	104.8 ± 2.67

Values are expressed as mean ± S.E.M. of 9 animals. Data analysis was carried out through unpaired Student's *t*-test. STZ – streptozotocin.

cleaved PARP/PARP and in the levels of caspase-3 in the hippocampus of mice experimentally induced by icv STZ. Apoptosis inhibition depends partly on the balance between the levels of Bcl-2 and Bax, increased apoptotic frequency is associated with decline in Bcl-2 expression (Upadhyay and Kamp, 2003). In other words, the decrease of Bcl-2/Bax ratio inhibits the DNA repair capacity with consequent disassembly and cell death (Pollack et al., 2002). Moreover, the increase of Bax/Bcl-2 ratio found in the hippocampus of icv STZ mice, can upregulate caspase-3 and increase apoptosis (Salakou et al., 2007). Another characteristic event of apoptosis is the proteolytic cleavage of PARP, a nuclear enzyme involved in DNA repair, DNA stability, and transcriptional regulation. Caspases, in particular caspase-3 and -7, cleave the 116-kDa form of PARP-1 to generate cleaved fragments (Wei and Shi, 2013). In the present study, ebselen reversed caspase- 3 activation and induction of PARP cleavage in the hippocampus of icv STZ mice. These findings, taken together, reinforce the anti-apoptotic action of ebselen in a sporadic model of AD.

The general consensus in the scientific literature is that adult hippocampal neurogenesis plays a vital role in the long-term spatial memory (Snyder et al., 2005) and that reduction of adult neurogenesis produces behavioral disturbances that lead to learning and memory impairment (Cameron and Glover, 2015). Whether decreased neurogenesis is just a neuroanatomical manifestation of the AD or functionally contributes to memory impairment remains uncertain, but it is known that neurogenesis can be regulated by numerous factors associated with behavioral intervention and cognitive states (Baptista and Andrade, 2018). In fact, behavioral interventions, including hippocampus dependent learning, environmental enrichment and voluntary running, can increase the rate of neurogenesis and BrdU positive cells (Deng et al., 2010).

Moreover, it is plausible that interventions that improve neurogenesis may be useful to treat hippocampal dysfunctions found in AD. Evidence found in transgenic mouse models of AD indicates that intrahippocampal transplantation of human neural stem cells improved cognition by enhancing synaptogenesis (Ager et al., 2015). Even though promising results highlight transplantation therapy as a possible intervention for AD it is not clear the role new neurons play in the functional activity of the mature brain and whether these cells display any clinical relevance (Apple et al., 2017).

In the present study, icv STZ reduced the neuronal cell proliferation in the dentate gyrus area of hippocampus, which helps to explain the memory impairment of STZ mice; however, neither ebselen nor donepezil was effective against this decrease even that they reversed memory impairment. This result leads us to believe that the multi target profile of ebselen is behind its memory enhancing effect demonstrated in this study.

Because ebselen is a bioavailable molecule that permeates the blood-brain barrier (Imai et al., 2001) it is possible that this drug acts largely through a direct central nervous system effects. Regarding the concentration of ebselen in plasma, a single oral dose of 100 mg/kg ebselen in rats produces serum values of 4–5 μM (Salom et al., 2004), whereas 1 mg/kg of ebselen (iv) reached 12 μg/ml in rat plasma (Imai et al., 2001). Moreover, data on ebselen pharmacokinetic reveal that the selenium portion was not bioavailable and, therefore, ebselen did not enter the selenium pool of the body. On the contrary, it was metabolized and excreted explaining its low toxicity (Parnham and Sies, 2000). Considering that ebselen has been reported to have antioxidant (Wang et al., 2014), anti-inflammatory (Xie et al., 2017) and neuroprotective (Singh et al., 2016) actions and that it inhibits the activity of acetyl cholinesterase (Martini et al., 2018) and phosphorylation of tau (Mahadevan et al., 2013), we may have found a new role for ebselen to treat diseases in which cognitive function is present.

This study has some limitations that have to be pointed out as follow, because neuronal atrophy is the main hallmark of AD (Fu et al., 2017), we acknowledge the lack of this end point measurement as a weakness of this study. The use of BrdU, a marker of cell proliferation; reflects on new neurons and glial cells. Although it is known that the granular layer of dentate gyrus consists predominantly of neurons to affirm that BrdU-positive cells are related to neuronal proliferation, it would be required a BrdU/NeuN co-labeling. There is no doubt that icv STZ is an experimental model to study metabolic sporadic AD and potential therapies. Nevertheless it is still an open question how is the STZ

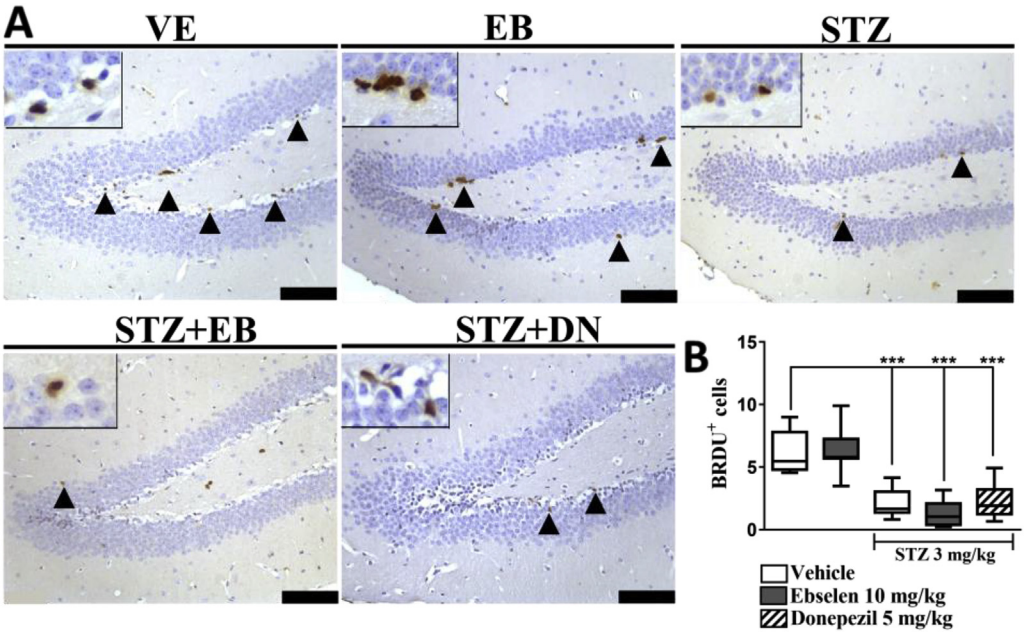

Fig. 7. Effect of ebselen on the number of BrdU positive cells in the dentate gyrus area of hippocampus in mice treated with STZ. Photographs are representation of qualitative immunohistochemistry (A) and BrdU$^+$ cells (B). Values are expressed as mean ± S.E.M. of 6–9 animals. Asterisk denotes the significance levels when compared with the respective vehicle treated group: (***) $P < 0.001$ (one -way ANOVA followed by the Newman Keuls).

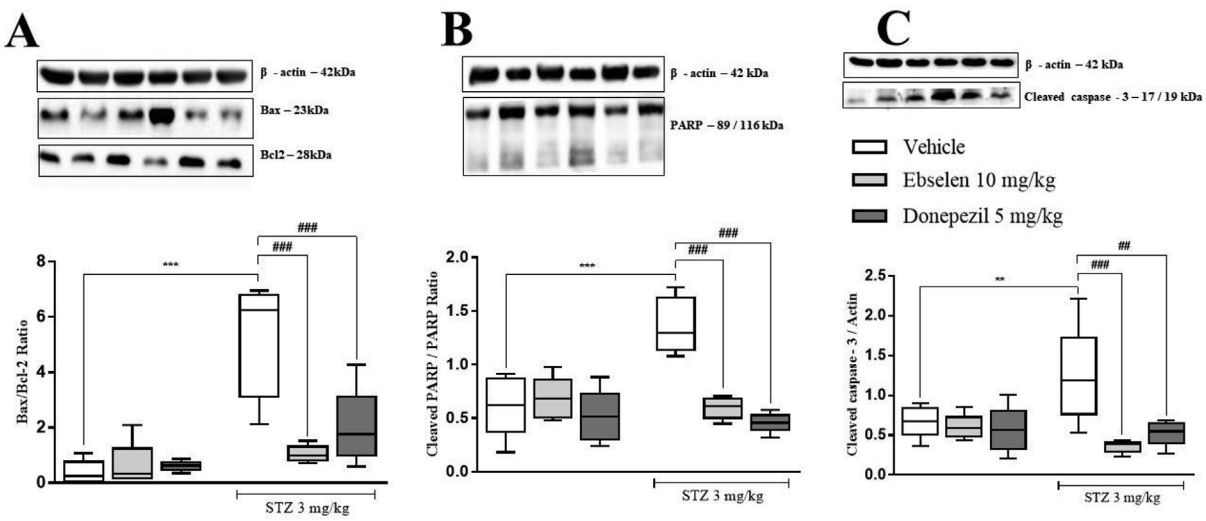

Fig. 8. Effect of ebselen on the hippocampal ratios of Bax/Bcl 2 (A) and cleaved PARP/PARP (C) and the levels of cleaved caspase- 3 (D) in mice treated with STZ. Values are expressed as mean ± S.E.M. of 5–6 animals. Asterisk denotes the significance levels when compared with the respective vehicle group: (*) $P < 0.05$, (**) $P < 0.01$ and (***) $P < 0.001$. Hashtag denotes the significance levels when compared with the STZ, vehicle treated group ($^{\#}$) $P < 0.05$, ($^{\#\#}$) $P < 0.01$ and ($^{\#\#\#}$) $P < 0.001$ (one -way ANOVA followed by the Newman Keuls).

mechanistic action, such as how is the brain insulin receptor desensitization or is brain glucose hypometabolism secondary effect or primary cause of AD (Grieb, 2016), these answers would help to converge the data obtained in this model to human sporadic AD. Moreover, it should be acknowledged the limitations of this model to mimic other relevant end points of sporadic AD.

Finally, in this study, most of ebselen effects, such as reversion of memory impairment, antioxidant and anti-apoptotic, were similar to those elicited by donepezil, the first-line treatment for Alzheimer's disease and non Alzheimer's dementia. Because long-term use of donepezil could be limited by increased vagal tone side effects associated to bradycardia, anorexia, abdominal pain, nausea and diarrhea (Turon-Estrada et al., 2003), drugs with fewer side effects would likely have a lower rate of people stop taking their prescribed drug.

In conclusion, the multifunctional selenoorganic compound, ebselen, was effective to reverse memory impairment, oxidative stress and apoptosis in a mouse model of metabolic sporadic AD induced by icv STZ.

Conflicts of interest

The authors declare they have no conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpsychires.2018.11.021.

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