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Research article

Oral treatment with royal jelly improves memory and presents neuroprotective effects on icv-STZ rat model of sporadic Alzheimer's disease

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ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive decline in cognitive function. Intracerebroventricular injection of streptozotocin (icv-STZ) has been used as an experimental model of Sporadic AD (SAD) in rodents and represents a promising tool for etiopathogenic analysis and evaluation of new therapeutic proposals for AD. The icv-STZ model shows many aspects of SAD abnormalities, resulting in decreased brain glucose and energy metabolism, cognitive impairment, oxidative stress, neuronal loss, and amyloid angiopathy. Royal jelly (RJ), a substance produced by worker honeybees of the *Apis mellifera* species, has been popularly used for more than 30 years in areas related to health eating and natural medicine. Researches indicate that RJ has a several pharmacological activities, including neuroprotective and improvement of cognitive function. The objective of this study was to investigate the effects of oral treatment with royal jelly during 2 weeks in Wistar rats submitted to icv-STZ on a working memory and neuroprotection, as evaluated by neurogenesis, neurodegeneration and oxidative stress. In this study, icv-STZ injection induced deleterious effects in the hippocampus, associated with cognitive impairments, and developed marked neurodegeneration, besides the reduction of neurogenesis and increased oxidative stress. On the other hand, RJ long-term oral administration induced beneficial effects in animals injured by icv-STZ injection, increasing retention time for working spatial memory, reducing neurodegeneration and oxidative stress level and increasing the proliferation of new neurons in the hippocampus. Thus, RJ promotes beneficial effects on cognitive functions and exhibits a neuroprotective action in the STZ experimental model of SAD.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive decline in cognitive and emotional functions, behavioral changes, judgment deficits and verbal fluency (Reitz et al., 2011). AD pathological manifestations include extracellular amyloid plaques containing amyloid beta protein aggregates (Aβ) and intracellular neurofibrillary tangles (NFT), composed by hyperphosphorylated tau protein, accompanied by activation of microglia, reactive astroglia, dystrophic neuritis, death of neurons and loss of synapses, especially in the entorhinal cortex, hippocampus, cerebral cortex and amygdala (Serrano-Pozo et al., 2011). These classic neuropathological changes contribute to the neurodegenerative process in AD affecting synaptic activity and plasticity with detrimental consequences for normal neuronal function. Certainly, dysfunction and synaptic loss are among the most well known changes correlated with memory deficit and cognitive impairment (Galeano et al., 2014).

Some findings on the pathophysiology of AD, including reduced brain glucose utilization, mitochondrial dysfunction, reduced ATP production, and energy dysregulation, led to the hypothesis that these abnormalities were mediated by desensitization of neuronal insulin receptors (Duelli et al., 1994; De La Monte et al., 2006). There have been studies showing that brain insulin and its receptors are functionally linked to cognitive performance, particularly spatial memory due to regulation of insulin mRNA in the hippocampus and increase of insulin receptors in hippocampal synaptic membranes (Zhao et al., 1999, 2004). All this

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information supports the hypothesis that insulin-deficient signaling plays an important role in AD pathogenesis (De La Monte and Wands, 2008).

Streptozotocin (STZ) injected intracerebroventricularly (icv) has been used as an experimental model to induce insulin resistance in the brain (Duelli et al., 1994; Lannert and Hoyer, 1998). STZ-icv administration induces changes in brain insulin receptors and their signaling, resulting in insulin-resistant brain state, with decreased cerebral glucose metabolism (Chen et al., 2013), thus leading to behavioral changes, neurochemical, morphological and histological characteristics similar to brain aging (Salkovic-Petrisic et al., 2006).

Santos and collaborators (Santos et al., 2012) reported disruption in working memory as early as 3 h after icv-STZ injections, accompanied by degenerative processes in the hippocampus valuated both 1 and 15 days after STZ injection. Moreover, these authors also showed that memory disruption increases over time and culminates with significant changes in amyloid-beta peptide and hyperphosphorylated Tau protein levels in distinct brain structures. Major neurochemical and structural changes have been observed in the first two weeks following icv-STZ administration, which persist for 12 weeks, accompanied by progressive and long-term deficits in learning and memory (Lannert and Hoyer, 1998; Grunblatt et al., 2007). Other studies also indicate that insulin resistance in the brain of icv-STZ treated rats decreases brain glucose metabolism, causes cholinergic deficits, oxidative stress, reactive gliosis, phosphorylation of Tau protein and amyloid beta peptide (Aβ) accumulation, in addition to memory and learning deficit (De La Monte, 2014).

Royal jelly (RJ), a substance produced by the hypopharyngeal glands of worker honeybees of the *Apis mellifera* L. species, has shown significant neuroprotective actions (Pavel et al., 2011). In the *Apis mellifera* L. honeybee species, queen bees are fed their whole life with RJ and worker bees receive this food for a short period during the larval stage of life. Queen bees live for 1–5 years, but worker bees, which are derived from the same diploid genome, live only for 3–6 weeks. In addition to living longer, queen bees are twice as large, have specialized anatomy for reproduction, and develop faster between egg and adult phases. These distinctions are derived from different conditions in the environment of larvae breeding, and especially of nutrition. This scenario raises the possibility that the royal jelly has a queen's longevity-promoting agent (Page and Peng, 2001; Honda et al., 2011).

Researches have shown that RJ has many pharmacological activities, among them antioxidant, neurotrophic, anti-inflammatory, immunomodulatory, hypoglycemic, antiallergic, general tonic and antiaging (Pavel et al., 2011; Teixeira et al., 2017; Pan et al., 2018; You et al., 2018; See Cornara et al., 2017, Pasupuleti et al., 2017 and Kocot et al., 2018 for review). Moreover, RJ long-term administration can affect the brain neurotransmitters in naturally aged rats (Pyrzanowska et al., 2014, 2018). In addition to experimental data, references indicate the medical use of RJ (Bogdanov, 2014). In Cuba, bee products, including RJ, are used within the official system of Natural and Traditional Medicine. In Russia, positive results have been found in a local hospital following treatment for several diseases with RJ (Salman, 2017).

Royal jelly has a potent ability to improve insulin resistance and this is a valuable effect in cases of AD (Zamami et al., 2008). Studies have shown that RJ has insulin-like activity, and contains several peptides similar to insulin (Münstedt et al., 2009). In addition, RJ extract facilitates neurogenesis by increasing the differentiation of neural stem cells into different types of brain cells, including neurons (Hattori et al., 2007). On the other hand, the same study also demonstrated that 10-hydroxy-2--decenoic (10-HDA), a component of RJ, increased the generation of neurons and decreased that of astrocytes. When administered orally, RJ has played neurotrophic and neuroprotective roles in the hippocampus of the adult rat brain (Hashimoto et al., 2005). It has been shown that RJ selectively facilitates gene expression at the mRNA level of the Glial Cell Derived Neurotrophic Factor (GDNF), a potent neurotrophic factor in the brain and the neurofilament H (NF-H), a specific marker predominantly found in the hippocampal neurons of the adult rat brain (Hashimoto et al., 2005). RJ oral administration has also been shown both to prevent trimethyltin induced acute neurodegeneration and to increase the number of granule cells in the dentate gyrus, with concurrent improvement of cognitive functions (Hattori et al., 2011). Together, these data suggest that oral administration of RJ may be a promising tool for enhancing neuronal function and regeneration of granular cells of the dentate gyrus, thus improving memory and cognition processes.

The present study investigated neuroprotective and behavioral effects of royal jelly long-term oral consumption in rats submitted to icv-STZ injection. This was achieved by analyzing (a) learning and spatial memory in a working memory version of the Morris Water Maze task; (b) anxiety and exploratory behavior using the Elevated Plus Maze; (c) neurodegeneration evaluated by the Fluoro-Jade B histochemistry; (d) oxidative stress measured by the superoxide anion index; (e) neurogenesis by analyzing the differentiation and survival of dentate gyrus granule cells; and (f) blood glucose levels.

2. Material and methods

2.1. Animals

Male Wistar rats weighing 220–250g were maintained in a room with constant temperature (22 ± 1 °C) on a 12 h light/dark cycle (lights on at 7:00 a.m.). Food and water were provided *ad libitum*. To avoid influences of female hormonal cycle we chose to use only male rats. In total, 127 animals were used in this study. It was performed according to National Institutes of Health guidelines and approved by the Animal Use Ethics Committee of the Butantan Institute (protocol nº.1345/14).

2.2. Royal jelly treatment

In natura Royal jelly (RJ) was obtained from the beekeeping company "Estação do Mel" (Pindamonhangaba - SP, Brazil). In all the study a RJ sample from the same lot was used. The RJ was kept at -80 °C. During the experiments it was conditioned in Eppendorf tubes in fractions of 350mg and kept at -20 °C. The RJ daily dose of 200 mg/kg (Dieamant, 2003) was administrated by oral gavage (og) along fourteen consecutive days from the 7th day after surgery. The duration of the treatment was based on references that indicated the period required for the observation of the RJ effects (Zamani et al., 2012). The RJ daily dose was thawed and diluted in distilled water (100 mg/ml). Rats from all groups remained fasted for 6 h (7h–13h) before receiving either RJ or distilled water (Control group).

2.3. Surgery for intracerebroventricular injection

The animals were anesthetized with ketamine (130 mg/kg) and xylazine (13 mg/kg). The Bregma was used as reference point for coordinates defined by the stereotaxic coordinate atlas (Paxinos and Watson, 1998). In order to confirm the correct location of the injection into the lateral ventricles, in a pre-study a group of animals (N = 10) received an icv injection of trypan blue followed by an immediately sacrifice, brain removal, freezing, and slicing at 40um (Figure 1).

Bilateral icv injections were performed using a 5μL Hamilton microsyringe The streptozotocin solution (3.0 mg/kg, b.w., Sigma, St Louis, MO (Salkovic-Petrisic et al., 2013)) was prepared immediately before use by diluting the drug in physiological Ringer's solution. A volume of 3.0 μL was injected in each hemisphere. The icv injection was performed using a flow of 1μl/min. After the injection, the needle was maintained in position for another 2 min. The animals of control groups underwent the same surgical procedures but received icv injection of physiological Ringer's solution. After surgical procedure, the animals were maintained in individual cages with food and water at a recovery period of 3 days.

Figure 1. Intracerebroventricular injections of trypan blue administered in a pre-study group (N = 10) to confirm the precise location of the injection in the lateral ventricles.

2.4. Experimental groups

The animals were organized into four experimental groups and received:

Control Group (CTR) - Ringer's solution (icv) and distilled water (og).
Streptozotocin Group (STZ) - Streptozotocin (icv) and distilled water (og).
Royal Jelly Group (RJ) - Ringer solution (icv) and royal jelly (og).
Streptozotocin Group - Royal Jelly (STZ-RJ) – Streptozotocin (icv) and royal jelly (og).

2.5. Behavioral tests

2.5.1. Morris Water Maze

A circular pool (200 cm diameter), painted with a black background, with water at a temperature of approximately 25 °C (Morris, 1984) was used. A transparent acrylic platform (10 cm diameter), placed 2 cm below the surface of the water, served as an escape for the animals. The pool was theoretically divided into 4 quadrants (Q1, Q2, Q3, Q4). After introducing the animal into the pool facing the wall, it could navigate for 120 s, with visual reference to the outer tracks hanging on the room. After finding the hidden platform, the animal remained on it for 15 s, ending that trial. When the rat did not find the hidden platform, the experimenter conducted it towards the platform where the animal remained for 15 s (Agrawal et al., 2009). In each session (one session per day) the animals were submitted to four trials. The intertrial interval (ITI) was standardized in two categories: ITI = 15min and ITI = Zero. The two intertrial interval were tested in independent experiments. In the first experiment the animals of the four groups (CTR (n = 7); RJ (n = 8); STZ (n = 7); STZ-RJ (n = 9) were tested using ITI15 during which the animal was maintained in a "waiting box", and in the second experiment the subjects (CTR (n = 6); RJ (n = 6); STZ (n = 7); STZ-RJ (n = 7) were tested using virtually "zero" intertrial interval (ITI0); in this latter case, the animal was returned to the pool immediately after the end of the previous trial. The scores used to analyze the animals performance included latency to find the platform after the animals release into the pool (LAT), distance (path length) and percentage of time spent within the critical counter (an area 30 cm in diameter surrounding concentric with the platform) (% T-A_CONT). The experiment was recorded by a video camera connected to a computer configured with the software Maze HVS-Image (version 2014) programmed to analyze pairs of coordinates that define the position of the animal collected every 0.1s. To avoid circadian effects on animal behavior, all behavioral tests were performed in the afternoon period, between 2:00 p.m. and 6:00 p.m.

2.5.2. Elevated Plus Maze

Each arm of the maze measures 45 cm long by 10 cm wide. The closed arms, opposite to each other, have walls 40 cm high, and the open arms, opposite to each other, have walls 1 cm high. The cross structure remained elevated at 70 cm from the floor. In the test session, each animal was released in the center of the equipment facing one of the open arms and observed for 5 min (Graef et al., 1993; Silva and Frussa-Filho, 2000). Anxiety and exploratory activity were evaluated using the percentage of entries into open arms (%EOA), percentage of entries into closed arms (%ECA), percentage of time spent within the open arms (%TOA), percentage of time spent within the closed arms (%TCA) and percentage of time spent on the central square (%TCS). In addition, these scores allowed to calculate the percentage of time spent within the open arms (%TOA), which is usually considered to inversely correlate with anxiety levels. The number of entries into closed arms is usually related to the level of motor activity of the animals. This experiment was run 15 days after the surgical procedures for icv-STZ injection, i.e., the 8th of the RJ administration, between 2:00 p.m. and 6:00 p.m. Before each test session, the apparatus was cleaned with 5% ethanol to avoid interference of olfactory cues produced by the other rats into the maze.

2.6. Histological analysis

2.6.1. Neurodegeneration

Fluoro-Jade B method was used for labeling hippocampal neurodegeneration (Schmued and Hopkins, 2000). The animals were deeply anesthetized with xylazine (1.5 mg/100 g) and ketamine (15 mg/100 g) and were perfused intracardially with 300 mL of 0.1M phosphate buffered saline (PBS), pH 7.4, followed by 300 ml of 4% paraformaldehyde in 0.1M phosphate buffer (PB), pH 7.4. The brain was removed from the skull and post-fixed for 4h in the same fixative at 4 °C and cryoprotected with 30% sucrose solution (PB) for 48 h at 4 °C. Coronal sections (30 μm) of the hippocampus region were cut on a cryostat (-21 °C) between the coordinates -1.8 mm and -4.8 mm from the Bregma (Paxinos and Watson, 1998). The gelatinized slides containing the brain slices were dried (37 °C) and rehydrated by immersion in 100% alcohol (3 min), 70% alcohol (1 min) and distilled water (1 min). After rehydration, the material was incubated in a solution of 0.06% potassium permanganate for 15 min under stirring to reduce background noise. Then, the material was incubated protected from light in 0.001% Fluoro-Jade B (Chemicon) solution and 0.1% acetic acid for 30 min. After incubation with Fluoro-Jade B, the material was washed 3 times in distilled water (1 min each) and left for 30 min in an oven to dry, and then treated two times with xylol for 3 min each, and covered with coverslip using DPX (Fluka, Milwaukee, WI, USA). The material was analyzed under a fluorescence microscope to capture green fluorescence (495–521nm). Digital images were captured using a 10x objective from a microscope (Nikon E1000, Melville, NY, USA) coupled to the camera (Nikon DMX1200). For neuronal death analysis, counts of the cell bodies of degenerating neurons were performed using Image J® 1.49v software (Wayne Rasband, National Institutes of Health, USA) in areas (480,000 μm^2) located in the regions of the dentate gyrus, CA1 and CA3 of the hippocampus, entorhinal cortex and striatum. The cell counts were performed in six sections of the brain of each animal considering the right and left hemispheres.

2.6.2. Oxidative stress

The oxidative stress was evaluated by the superoxide anion levels (O2 • -) in the dentate gyrus (DG), measured by the oxidation index of the dihydroethidium (DHE) (Sharikabad et al., 2001).The animals were sacrificed by decapitation and their brains were immersed in tissue freezing medium (Leica Instruments) and then frozen on dry ice. Coronal

sections (30 μm) were made in cryostat (-21 °C) in the hippocampus region between the coordinates -1.8 mm and -4.8 mm from Bregma (Paxinos and Watson, 1998). The slides containing the sections were kept frozen at -20 °C until use. The sections were thawed in hot plate (37 °C) for 10 min and then circled with a hydrophobic pen. Tissue sections were incubated with 0.1 M phosphate buffer (PB) containing diethylenetriaminepenta acetic acid (DTPA) (100 μM) (10 min) to prevent secondary oxidation reaction artifacts and then incubated with diluted DHE (5 μM) in PB containing 100 μM DTPA in a humid chamber (5 min) at room temperature, protected from light. The fluorescence of the slices was detected in a Nikon optical microscope with a rhodamine filter (480.586nm), with a 10x objective. Digital images were obtained with a camera coupled to the microscope. The parameters used to capture the digital images were kept constant. The fluorescence was analyzed by the measurement of quantity and optical intensity of the labeling in an area of 480,000 μm^2 in the CA1, CA3 and dentate gyrus (DG) regions and in an area of 240,000 μm^2 in the hilus region. Automated analysis was performed by Image J® 1.49v software (Wayne Rasband, National Institutes of Health/USA). Quantifications were performed unilaterally (right or left hemisphere) in six sections of the brains of each animal.

2.6.3. Neurogenesis

Neurogenesis was assessed by the proliferation and survival of cells in the dentate gyrus (DG). The animals were deeply anesthetized with xylazine (1.5 mg/100 g) and ketamine (15 mg/100 g) and were perfused intracardially with 300 ml of 0.1M phosphate buffered saline (PBS), pH 7.4, followed by 300 mL of 4% paraformaldehyde in 0.1M phosphate buffer (PB), pH 7.4. The brain was removed and post-fixed for 4h in the same fixative at 4 °C and cryoprotected with 30% sucrose solution (PB) for 48 h at 4 °C. Coronal sections (30 μm) were cut using a cryostat (-21 °C) in the hippocampus region between the coordinates -1.8 mm and -4.8 mm from the Bregma (Paxinos and Watson, 1998). For labeling cell proliferation, doublecortin marker (DCX) was used, which identifies immature neurons under development (Mcdonald and Wojtowicz, 2005). Immunohistochemistry for DCX was performed in these sections according to the method of (Diniz et al., 2013). The sections were washed by shaking in 0.1M PB solution, pH 7.4 (3 × 10 min) and then preincubated for 1h in 10% normal donkey serum (NDS) solution (Jackson Immuno Research Lab, USA) diluted in 0.1 M phosphate buffer (PB) containing 0.3% Triton X-100 to avoid non-specific binding. The sections were washed again and subsequently incubated with goat polyclonal primary antibody (1:100, goat anti-DCX, Santa Cruz Biotechnology Inc., USA), for 48 h. After primary incubation, the sections were washed and then incubated with a secondary antibody (1:200, donkey anti-goat, Jackson Immuno Research Lab, USA) in 0.1 M PB containing 0.3% Triton X-100 for 2 h, followed by another wash. Then the immunoperoxidase protocol was applied (Diniz et al., 2013). The labeling for new surviving neurons in the DG was performed using BrdU (5-bromo-2′-deoxyuridine) labelling. On the fourteenth day after icv-STZ injection (thus, seven days after starting oral administration of royal jelly) the animals received two intraperitoneal administrations of Brdu (Sigma, St. Louis, MO, USA) at a dose of 200 mg/kg each, with an interval of 12 h between them, to maximize labelling of dividing cells. Twenty-one days after administration of the Brdu, the animals were anesthetized, transcardially perfused, the brains being removed from the skull, post-fixed and sliced as previously described. For Brdu detection, the material was initially treated/denatured with 2N HCl for 1 h. Then, after 3 washes of 10 min each in PBST, the material was treated with 0.1M sodium tetraborate (Na2B4O7), at 4 °C, for 10 min, and washed with PBST (3 × 10min) (Girardi et al., 2002). The brain slices were preincubated overnight with 30% normal goat serum and subsequently incubated with rat anti-BrdU monoclonal antibody (Sigma, St. Louis, MO, USA), diluted 1:200 in PBST, for 48 h at room temperature. After three washes in PBST (3 × 10 min), the sections were incubated with goat anti-mouse secondary antibody (Jackson Immuno Research Lab., Pennsylvania, USA) diluted 1:200 in PBST, for 2 h. Subsequently, the immunoperoxidase protocol was performed (Diniz et al., 2013). Digital images were captured using a 10x objective from a light microscope (Nikon E1000, Melville, NY, USA) coupled to a camera (Nikon DMX1200), and an area of 480,000 μm^2, containing labeled cells located in the region of the DG was analyzed. Counts of cell bodies of DCX-positive neurons were performed using Image J® 1.49v software (Wayne Rasband, National Institutes of Health, USA). The cell counts were performed in six sections of the brain of each animal, considering the right and left hemispheres.

2.7. Peripheral blood glucose levels

With the aid of sterile surgical scissors, a drop of blood was obtained through the piercing of the tail of the animals. This blood sample was applied to a test strip which was introduced in a glycosimeter (Accu-Chek Active, Roche). Results were given as mg/dL.

2.8. Body weight

The rats were weighed weekly from the day of icv-STZ injection surgery (considered day "zero" in the time line), and the variation of weights calculated as the percentage of variation with respect to the weight measured in the surgery day.

2.9. Mortality rate

The percentage mortality rate of the animals was calculated at the end of the test period.

2.10. Experimental design

Independent groups of rats were used along this study. Among the animals that underwent behavioral tests (Morris Water Maze ITI = 15 min and Elevated plus-maze), those that were sacrificed on the 21st day after the surgery procedure had brain processed for histological analysis of neurogenesis – cell proliferation (DCX) and neurodegeneration (Fluoro-Jade). The subjects that were sacrificed on the 35th after surgery procedure had brain processed for histological analyses of neurogenesis – cell survival (Brdu). A third group of rats was used for the oxidative stress analysis (DHE). In addition, a fourth group of animals underwent behavioral analysis in the Morris Water Maze ITI = zero did not have histological analyzes associated with this study (Figure 2).

2.11. Statistical analysis

Statistical analysis of behavioral data in the Morris Water Maze involved a three-way Analysis of Variance (ANOVA), having groups as the between-subjects factor and session and trial within-subjects factors, followed by Bonferroni post-test, using the IBM SPSS Statistics v23 software. Statistical analysis of data obtained in the Elevated Plus Maze and the histological analyzes of neurogenesis, neurodegeneration, oxidative stress, glycemia, body weight and mortality rate, involved a two-way ANOVA, and followed by the post-test Bonferroni using of GraphPadPrism v5 software. The level of significance considered as significant was set at 5% ($P < 0.05$).

3. Results

3.1. Learning and memory in the Morris Water Maze Test – (ITI15)

The results of the working memory evaluated by Morris water maze test on the pre-STZ injection training (**Period 1**, when the subjects had already been ascribed to different groups but they had not yet received any treatment) in terms of Latency (LAT), Distance (DIST) and % time within the critical counter (% T-A_CONT) are shown in Table 1 and Figure 3. As expected, the ANOVAs for these scores revealed significant Trial [$F_{3,492} = 2.75–64.69$; $p < 0.05$) and Session [$F_{3,492} = 5.99–49.32$; p

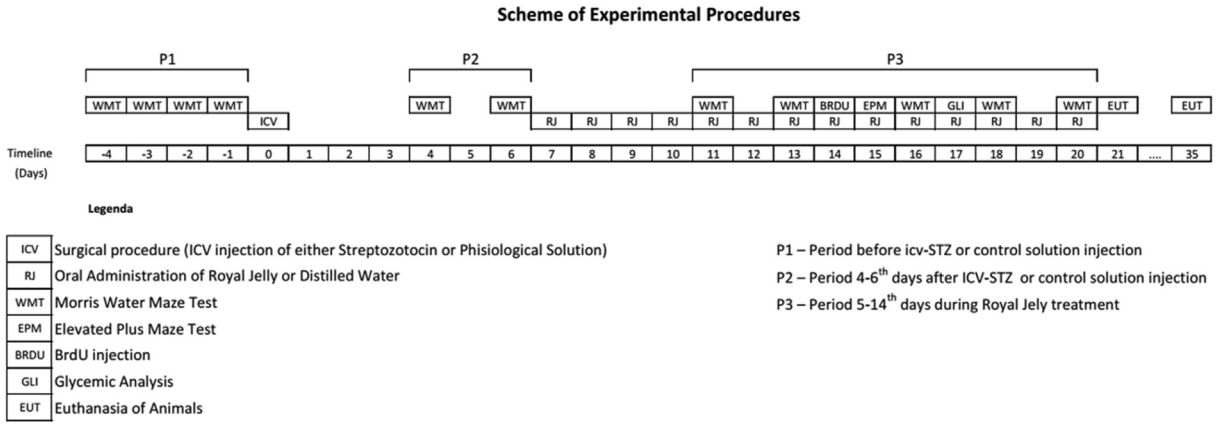

Figure 2. Flow chart showing the Experimental Design. P1 Period – The subjects were ascribed to four different groups: Control Group (CTR); Streptozotocin Group (STZ); Royal Jelly Group (RJ); Streptozotocin-Royal Jelly Group (STZ-RJ) but none of them had received any treatment in this period (pre-surgery period) when the animals were submitted to Water Maze (WM) task. Day Zero corresponding the surgery day when all animals of the four groups were submitted to icv injection of STZ or Ringer's solution. P2 Period corresponding the interval between the 4th-6th days after the surgery when the subjects were submitted to WM task to available the effects of icv-STZ injection. Day 7 on the timeline corresponding the beginning of the Royal Jelly treatment that was extended until Day 21 (RJ long-term treatment for fourteen days). P3 Period comprising the 5-14th days of the RJ treatment when the subjects were submitted to WM task and elevated Plus maze (EPM) test to available the effects of the Royal Jely treatment on rats icv-STZ injected or Ringer's solution. On Day 21 the subjects were euthanatized and the brains were processed to analyse the neurodegeneration or oxidative stress, except the animals of the group selected to receive BrdU on the 14th day of the timeline that were used to analyse the Neurogenesis and were euthanatized on Day 35. For more details see Material and Methods.

Table 1. Working memory evaluated by Morris water maze test on the pre-STZ injection training with an intertrial interval of 15 min (Period 1 - ITI 15).

Parameters	Group		Trial		Trial/Group	
	F 3,492	*P*	F 3,492	*P*	F 3,492	*P*
LAT	—	—	49,48	<0,001	—	—
DIST	—	—	40,934	<0,001	—	—
%T-A_CONT	—	—	29,173	<0,001	—	—

Parameters	Day		Day/Group		Day/Trial		Day/Trial/Group	
	F 3,492	*P*	F 3,492	*P*	F 3,492	*P*	F 3,492	*P*
LAT	18,287	<0,001	—	—	—	—	—	—
DIST	16,951	<0,001	—	—	—	—	—	—
%T-A_CONT	21,827	<0,001	—	—	1,93	0,048	—	—

Result of the statistical analysis of behavioral data in the Morris Water Maze employing a three-way Analysis of ANOVA, having groups as the between-subjects factor and session and trial within-subjects factors, followed by Bonferroni post-test. The level of significance considered as significant was set at 5% ($P < 0.05$).

< 0.05] significant differences, and lack of significant differences for Groups [$F_{3,492} = 0.19–0.74$; $p > 0.53$]. These figures indicate that all subjects did learn to navigate within the water maze to find the escape platform, as their latencies and distances to reach it decreased and their percentage of time within the critical counter increased along trials and sessions. The results also show that performance in the "different" groups (note that this difference in the groups, in this phase, is only prospective, because the treatments with STZ and RJ were installed later) did not differ among each other, thus showing the equivalence of the behavioral performance before treatments.

The results of **Period P2**, shown in Table 2 and Figure 4, allow us to analyze the effects of the icv-STZ injection on the 4th and 6th day after the surgical procedures. Note that in this phase of the trials, it was evaluated only the effects of the icv-STZ or icv-CTR injection, since the royal jelly oral administration was initiated on the 7th day following surgery. The data show that for the parameters Latency (LAT), Distance (DIST) and % time within the critical counter (% T-A_CONT) the ANOVA revealed the existence of a significant effect for the Group Factor [F (3,244) = 4,83–14,31; $p < 0.05$], and for the Trial Factor [F (3,244) = 5,20–13,33; $p < 0.05$) was demonstrated in the same parameters. In Period P2 the ANOVA revealed no significant effect for the Day Factor [F (3,244) = 0.06–2.52; $p > 0.12$] indicating that the animals showed no change in performance over the test sessions within this period. The significant effect for the Trial Factor indicates that the animals learned the task and memorized the platform location throughout the trials. The significant effect for the Group Factor shows that there was a significant difference in learning between the groups. The Bonferroni post-test showed that the animals in the groups receiving icv injection of STZ (STZ and STZ-RJ) presented learning and memory losses when compared to the animals of the groups receiving icv injection of Ringer's solution (CTR and RJ) ($p < 0.05$).

The results of the Period P3, shown in Table 3 and Figure 5, allow us to analyze the effects of icv-STZ injection and the effects of RJ long-term administration on the performance of rats in working memory tasks in the Morris Water Maze Test. The data show that for the parameters Latency (LAT), Distance (DIST) and % time within the critical counter (% T-A_CONT) the ANOVA revealed the existence of a significant effect for the Group Factor [F (3,616) = 6,32–11,17; $p < 0.05$], and for the Trial Factor [F (3,616) = 16,73–41,66; $p < 0.05$]. The significant effect for the Trial Factor indicates that the animals learned the task and memorized the platform location throughout the trials. The ANOVA also revealed a significant effect for the Day Factor [F (3,616) = 5.99–49.32; $P < 0.05$) Latency, Distance and % time within the critical counter (% T-A_CONT), indicating that the animals presented improvement of performance

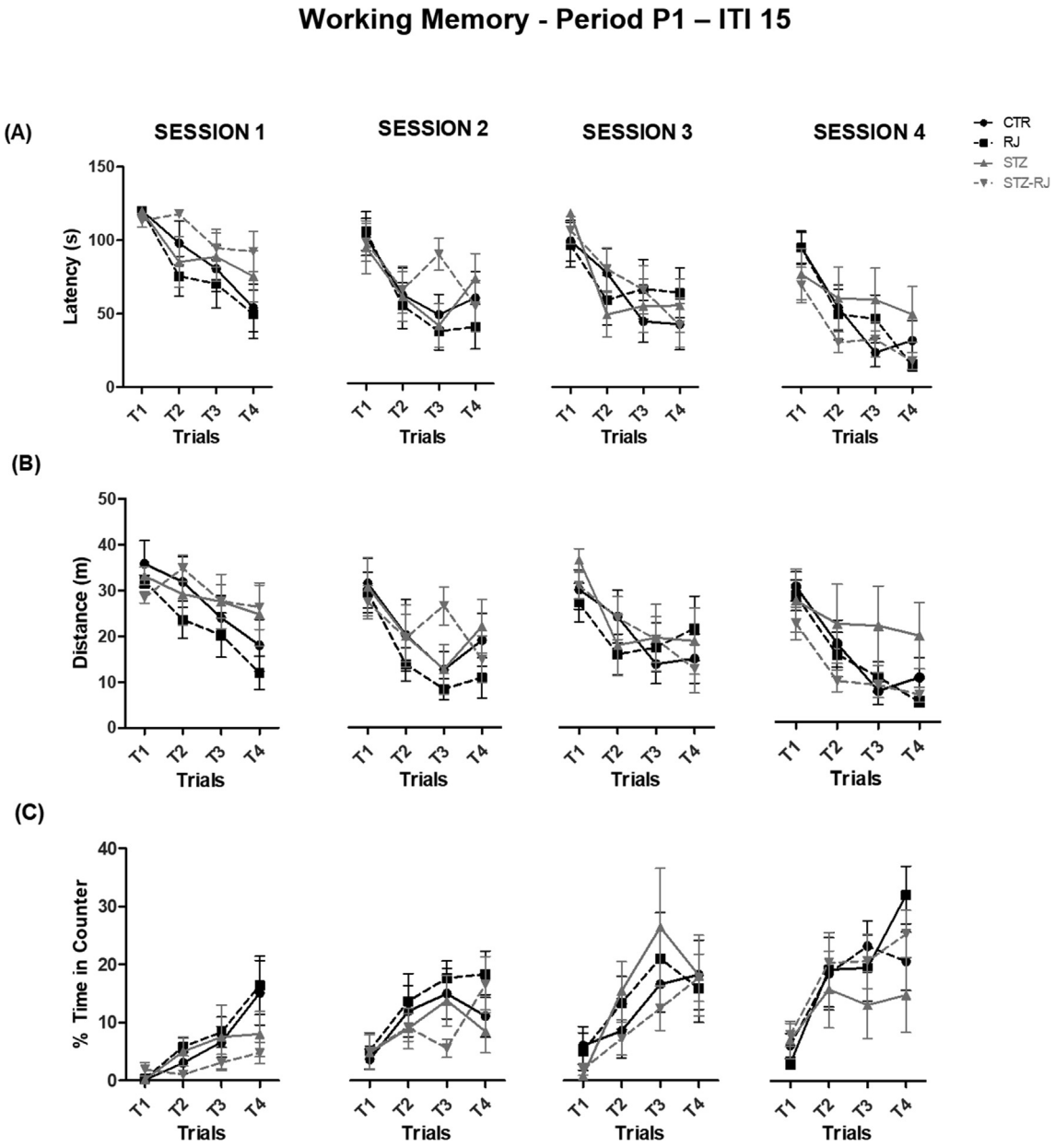

Figure 3. Performance of the animals throughout the sessions in terms of (A) Latency, (B) Distance and (C) % time within the critical counter (data expressed as mean ± standard error) in the Morris Water Maze Task along 4 Trials per session (the intertrial interval was 15 min - ITI15), and 4 Sessions (one Session per day). Groups (prospectively): CTR = Control (n = 7); RJ = Royal Jelly (n = 8); STZ = Streptozotocin (n = 7); STZ-RJ = Streptozotocin-Royal Jelly (n = 9).

throughout the test sessions performed within this period. The significant effect for the Group Factor shows that there was a difference in learning among the groups. The Bonferroni post-test showed that the animals in the groups receiving icv injection of STZ (STZ and STZ-RJ) presented learning and memory disruption when compared to the animals of the groups receiving a icv Ringer's solution injection (CTR and RJ) and that the oral treatment with RJ was not able to produce changes in the performance of the animals.

The Figure 6 presents an integrated view of the performance curve of the animals in the parameters Latency (LAT), Distance (DIST) and % time within the critical counter (% T-A_CONT) over the test sessions performed in the Period P1 (S1-S4), Period P2 (S5-S6) and Period P3 (S7-S11). It is possible to notice the equivalence of performance among the four experimental groups (CTR, RJ, STZ, and STZ-RJ) in the pre-surgery period, Period P1. In the P2 and P3 Periods, when evaluating the performance of the animals from the 4th to the 20th day after the icv injection surgery, it is possible to observe the performance reduction of the animals receiving the icv injection of streptozotocin (STZ and STZ-RJ) with respect to animals receiving icv injection of Ringer's solution (CTR and RJ). We can see that prolonged treatment with RJ did not alter the induced injury by STZ.

3.2. Learning and memory in the Morris Water Maze test – (ITI 0)

The results of the working memory evaluated by Morris water maze test using the intertrial interval equal to zero (ITI0) of related to the Period P1 are shown in Table 4 and Figure 7. The data indicate that for the parameters Latency (LAT), Distance (DIST) and % time within the critical counter (% T-A_CONT) the ANOVA revealed the existence of a significant effect for the Trial Factor [$F(3,412) = 3.83–47.90$; $p < 0.05$) and no significant effect for the Group Factor [$F(3,412) = 0,12–2,82$; $p > 0.06$]. The significant effect for the Trial Factor indicates that the animals presented a learning curve during the trials and the lack of effect of the Group Factor indicates that all groups presented similar performance,

Table 2. –Working memory evaluated by Morris water maze test on the 4th and 6th day after the icv injection of STZ or control solution with an intertrial interval of 15 min (Period 2- ITI 15).

Parameters	Group		Trial		Trial/Group	
	F 3,244	*P*	F 3,244	*P*	F 3,244	*P*
LAT	14,313	<0,001	7,579	<0,001	—	—
DIST	11,825	<0,001	5,209	0,002	—	—
%T-A_CONT	8,934	<0,001	9,811	<0,001	—	—

Parameters	Day		Day/Group		Day/Trial		Day/Trial/Group	
	F 3,244	*P*	F 3,244	*P*	F 3,244	*P*	F 3,244	*P*
LAT	—	—	—	—	—	—	—	—
DIST	—	—	—	—	—	—	—	—
%T-A_CONT	—	—	—	—	—	—	—	—

Result of the statistical analysis of behavioral data in the Morris Water Maze employing a three-way Analysis of ANOVA, having groups as the between-subjects factor and session and trial within-subjects factors, followed by Bonferroni post-test. The level of significance considered as significant was set at 5% ($P < 0.05$).

validating the equivalence between the groups before the surgical and treatment procedures with royal jelly. ANOVA also revealed a significant effect for Day Factor [F (3,412) = 2.94–31.69; $p < 0.05$) for Latency, Distance and %Time within the critical counter (LAT, DIST and %T-A_CONT), indicating that the animals improved performance throughout the daily test sessions performed within this period. When looking at Figure 10 it is possible to notice that all the groups presented similar learning curves, indicating performance improvement during the attempts made in the Period P1.

The results of Period P2, shown in Table 5 and Figure 8, allow us to analyze the effects of the icv-STZ injection on the 4th and 6th day after the surgical procedures. Note that in this phase of the trials, it was evaluated only the effects of the icv-STZ or icv-CTR injection, since the royal jelly oral administration was initiated on the 7th day following surgery. The results of Period P2 show that for the parameters Latency (LAT), Distance (DIST) and % time within the critical counter (% T-A_CONT) the ANOVA revealed the existence of a significant effect for the Group Factor [F (3,204) = 7,10–20,49; $p < 0.05$], and for the Trial Factor [F (3,204) = 5,20–19,10; $p < 0.05$]. In Period P2 the ANOVA revealed no significant effect for the Day Factor [F (3,204) = 0,03–1,15; $p > 0.86$] indicating that the animals showed no change in performance during the test sessions within this period. The significant effect for the Trial Factor indicates that the animals learned the task and memorized the platform location throughout the attempts. The significant effect for the Group Factor shows that there was a difference in learning between the groups. The Bonferroni post-test showed that the animals in the groups receiving icv injection of STZ (STZ and STZ-RJ) presented learning and memory disruption when compared to the animals of the groups receiving icv injection of Ringer's solution (CTR and RJ) ($p < 0.05$) (Figure 8).

The results of the Period P3, shown in Table 6 and Figure 9 allow us to analyze the effects of icv-STZ injection and the effects of prolonged administration of royal jelly on the performance of rats in working memory tasks in the Morris Water Maze. The data show that for the parameters Latency (LAT), Distance (DIST) and % time within the critical counter (% T-A_CONT), the ANOVA revealed the existence of a significant effect for the Group Factor [F (3,516) = 7.27–14.20; $p < 0.05$), and the existence of a significant effect for the Trial Factor [F (3,516) = 12,11–72,31; $p < 0.05$] was demonstrated by the same parameters. The significant effect for the Trial Factor indicates that the animals learned the task and memorized the platform location throughout the trials. In Period P3 the ANOVA revealed a significant effect for the Day factor [F (3,516) = 3,18–4,05; $p < 0.05$) for LAT, indicating that the animals showed improved performance throughout the test sessions within this period. The significant effect for the Group Factor indicates that there was a difference in learning between groups. The Bonferroni post-test showed that the animals in the groups receiving icv injection of STZ (STZ and STZ-RJ) presented learning and memory losses when compared to the animals in the groups that received icv-Ringer's solution injection (CTR and RJ) and that the oral treatment with RJ was able to improve the performance of the animals ($p < 0.05$). When looking at Figure 9, it is possible to notice that the animals of the STZ group presented reduced performance when compared to the other groups, and that the treatment with RJ produced a marked improvement in the performance of the animals of the STZ-RJ group and with statistical significance in the parameters LAT ($p < 0.05$).

The Figure 10 shows an integrated view of the performance variation of the animals in the parameters Latency (LAT), Distance (DIST) and % time within the critical counter (% T-A_CONT) during the test sessions performed in the Period P1 (S1-S4), Period P2 (S5-S6) and Period P3 (S7-S11). It is possible to note the performance equivalence between the four experimental groups (CTR, RJ, STZ, and STZ-RJ) in Period P1. In period P2, after the intracerebroventricular injection surgery, the performance of the animals receiving icv streptozotocin injection (STZ and STZ-RJ) in relation to the animals receiving icv injection of Ringer's solution (CTR and RJ) could be observed. In Period P3, during the oral treatment with royal jelly it is possible to observe the gradual improvement of the performance of the animals of the STZ-RJ group.

3.3. Anxiety and exploratory behavior in the Elevated Plus Maze

According to ANOVA, the results of the Elevated Plus Maze Test show that there were no significant differences in any of the observed parameters, neither in the STZ Factor [F (3,28) = 0,077–5,077; $p > 0.782$] nor the RJ Factor [F (3.28) = 0.070–1.72; $p > 0.792$), indicating that the icv-STZ injection and the RJ oral treatment did not produce changes in the anxiety levels and exploratory behavior of the animals (Figure 11).

3.4. Neurodegeneration

In the dentate gyrus (DG) and CA1 and CA3 areas of the hippocampus, no neurodegeneration labels were found in animals receiving icv injection of Ringer's solution (CTR and RJ groups). In the entorhinal cortex and striatum no neurodegeneration labels were observed in either group. In the DG, a significant effect of the STZ Factor was observed in the generation of neurodegenerative process [F (3,16) = 115,9; $p < 0.001$] and significant effect of the RJ Factor [F (3,16) = 7.836; $p = 0.01$], indicating that RJ was able to reduce levels of neurodegeneration caused by the icv-STZ injection. In the CA1 region, a significant effect of the STZ Factor was observed in the generation of the neurodegenerative process [F (3,16) = 30,08; $p < 0.001$], and no significant effect for RJ Factor [F (3,16) = 0.4121; $p = 0.53$], indicating that treatment with RJ was not able to alter the levels of neurodegeneration caused by the icv-STZ

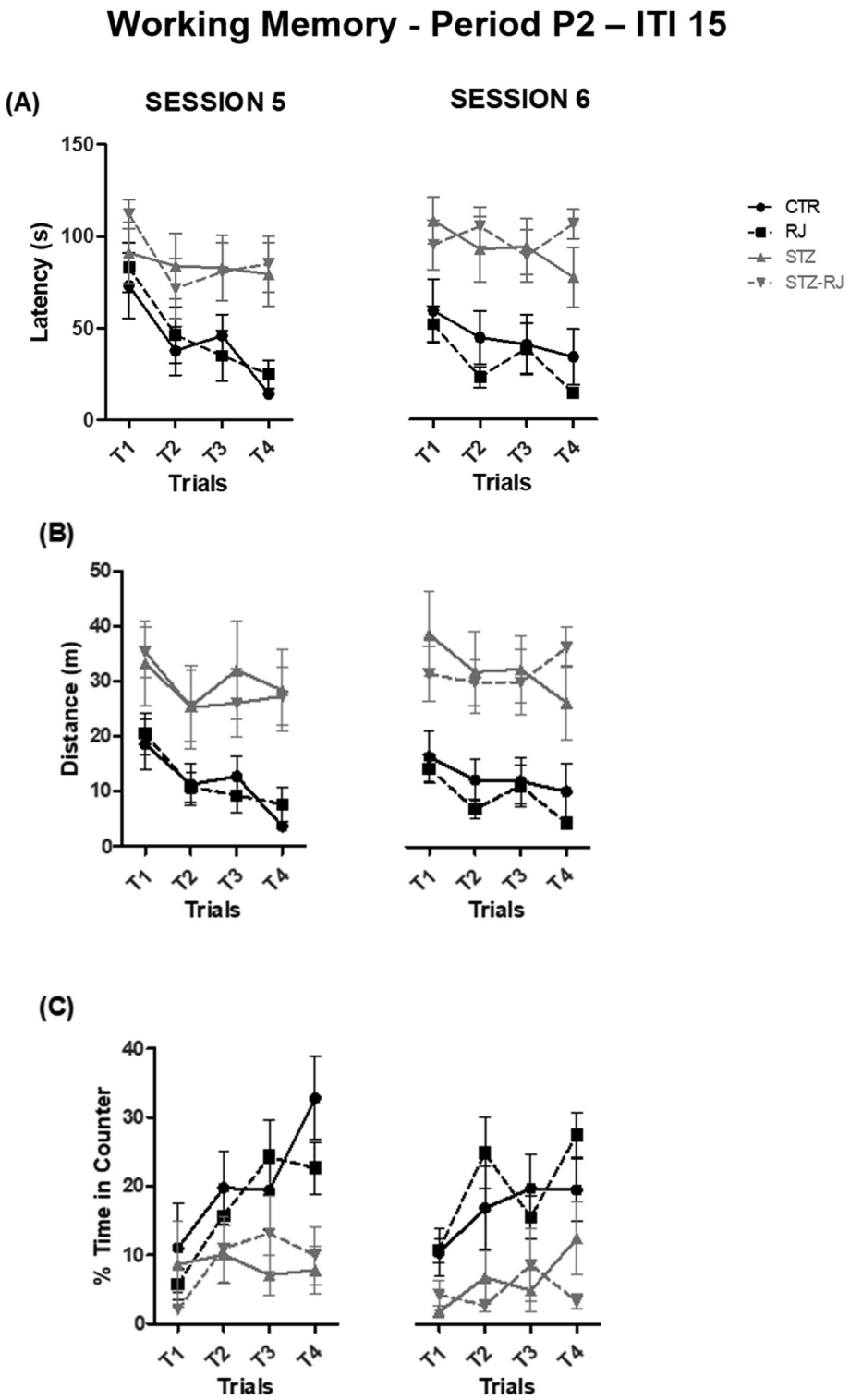

Figure 4. Performance of the animals throughout the sessions in terms of (A) Latency, (B) Distance and (C) % time within the critical counter (data expressed as mean ± standard error) in the Morris Water Maze Task along 4 Trials per session (the intertrial interval was 15 min - ITI15), and 2 Sessions (one Session per day). Groups: CTR = Control (n = 7); RJ = Royal Jelly (n = 8); STZ = Streptozotocin (n = 7); STZ-RJ = Streptozotocin-Royal Jelly (n = 9).

injection in CA1 region. In CA3, a significant effect of the STZ Factor was observed in the generation of neurodegenerative process [F (3,16) = 26,41; $p < 0.001$], and no significant effect for RJ Factor [F (3,16) = 0.0138; $p = 0.908$], indicating that treatment with RJ was not able to alter the levels of neurodegeneration caused by the icv-STZ injection in CA3 region. The results of the neurodegeneration are presented in Figure 12.

3.5. Oxidative stress

The oxidative stress was evaluated by the superoxide anion levels, measured by the oxidation index of dihydroethidium (DHE) in CA1, CA3, dentate gyrus (DG) and hilus regions. The results were accounted by the measurement both in terms of quantity and the optical intensity of labeling. In the DG, it was observed a significant oxidative increasing effect

Table 3. Working memory evaluated by Morris water maze test along the RJ long-term administration in rats icv injected with STZ or control solution with an intertrial interval of 15 min (Period 3 - ITI 15).

Parameters	Group		Trial		Trial/Group	
	F 3,616	*P*	F 3,616	*P*	F 3,616	*P*
LAT	10,865	<0,001	41,663	<0,001	4,378	<0,001
DIST	11,178	<0,001	38,468	<0,001	2,724	0,008
%T-A_CONT	8,732	<0,001	40,589	<0,001	4,844	<0,001

Parameters	Day		Day/Group		Day/Trial		Day/Trial/Group	
	F 3,616	*P*	F 3,616	*P*	F 3,616	*P*	F 3,616	*P*
LAT	3,589	0,009	—	—	—	—	—	—
DIST	5,532	<0,001	—	—	—	—	—	—
%T-A_CONT	2,801	0,029	—	—	1,989	0,025	—	—

Result of the statistical analysis of behavioral data in the Morris Water Maze employing a three-way Analysis of ANOVA, having groups as the between-subjects factor and session and trial within-subjects factors, followed by Bonferroni post-test. The level of significance considered as significant was set at 5% ($P < 0.05$).

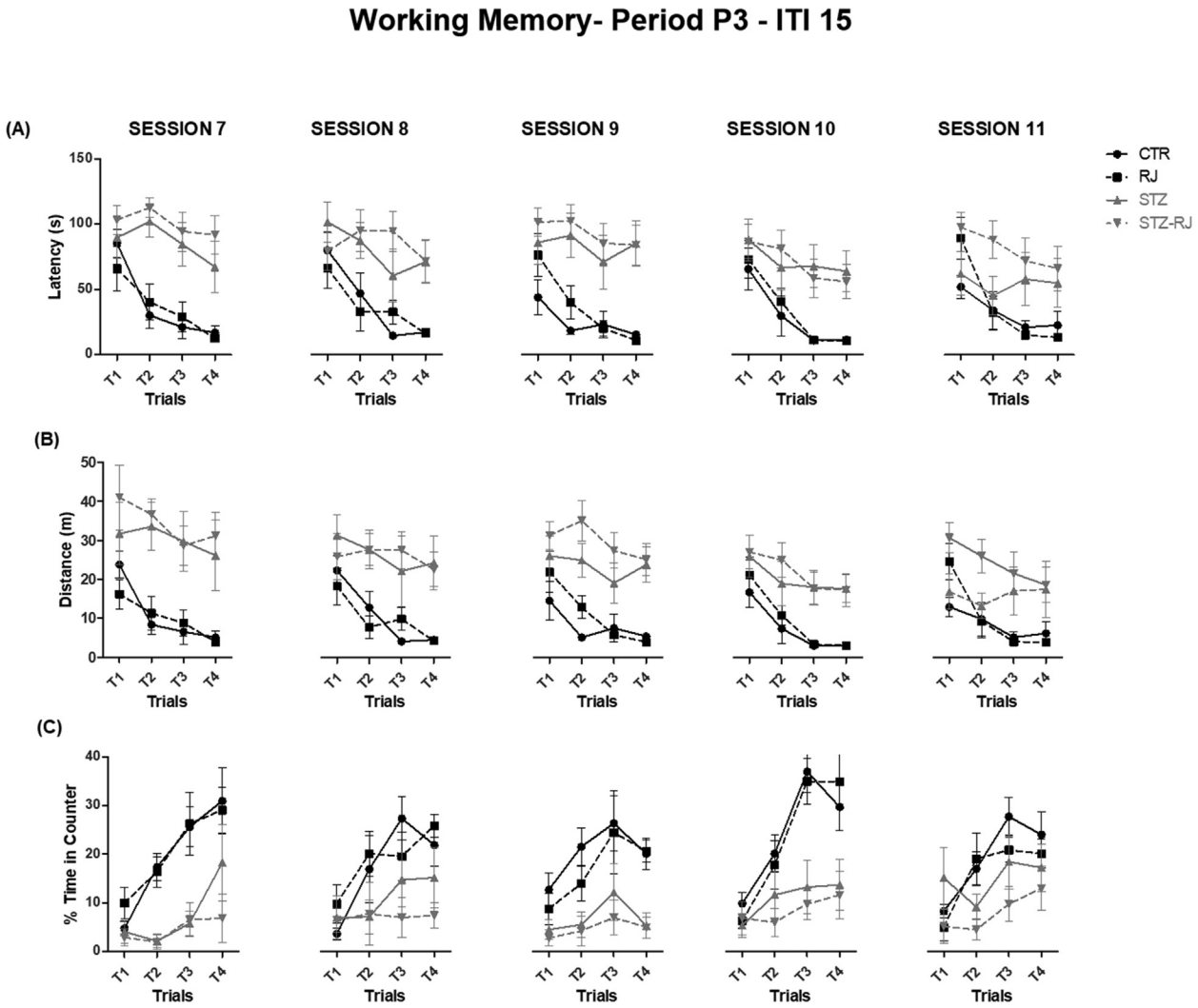

Figure 5. Performance of the animals throughout the sessions in terms of (A) Latency, (B) Distance and (C) % time within the critical counter (data expressed as mean ± standard error) in the Morris Water Maze Task along 4 Trials per session (the intertrial interval was 15 min - ITI15), and 5 Sessions (one Session per day). Groups: CTR = Control (n = 7); RJ = Royal Jelly (n = 8); STZ = Streptozotocin (n = 7); STZ-RJ = Streptozotocin-Royal Jelly (n = 9).

of the STZ Factor [F (3,15) = 8,904; p = 0.008] and a non-significant effect of the RJ Factor [F (3,15) = 0,3512; p = 0.5608] on the number of labeled nuclei. When the optical intensity of labeling was analyzed, it was verified a significant increasing oxidative effect of the STZ Factor [F (3,15) = 10,06; p = 0.0063] and a tendency of a significant decreasing oxidative effect of the RJ Factor [F (3,15) = 3,924; p = 0.0662]. The trend of a decreasing effect of the RJ treatment on the oxidative stress levels, measured by the optical intensity of the labeling, can also be observed when comparing the percentage difference between groups. When comparing the CTR group with RJ group, a reduction of 61.9% in

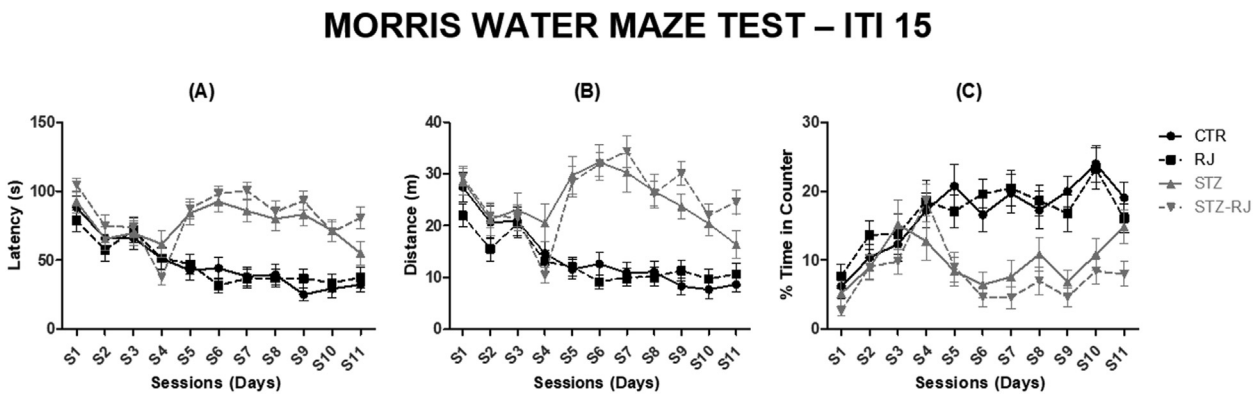

Figure 6. Performance of the animals throughout the sessions in terms of (A) Latency, (B) Distance and (C) % time within the critical counter in parameters in the Periods P1 (S1-S4), P2 (S5-S6) and P3 (S7-S11) (data expressed as mean ± standard error) in the Morris Water Maze Task along 4 Trials per session (the intertrial interval was 15 min - ITI15) and 11 Sessions (one Session per day). Groups: CTR = Control (n = 7); RJ = Royal Jelly (n = 8); STZ = Streptozotocin (n = 7); STZ-RJ = Streptozotocin-Royal Jelly (n = 9).

Table 4. Working memory evaluated by Morris water maze test on the pre-STZ injection training with an intertrial interval of 0 min (Period 1 - ITI 0).

Parameters	Group		Trial		Trial/Group	
	F 3,412	*P*	F 3,412	*P*	F 3,412	*P*
LAT	—	—	40,214	<0,001	—	—
DIST	—	—	32,174	<0,001	—	—
%T-A_CONT	—	—	15,278	<0,001	—	—

Parameters	Day		Day/Group		Day/Trial		Day/Trial/Group	
	F 3,412	*P*	F 3,412	*P*	F 3,412	*P*	F 3,412	*P*
LAT	21,018	<0,001	—	—	—	—	—	—
DIST	31,699	<0,001	—	—	2,618	0,007	—	—
%T-A_CONT	2,941	0,04	—	—	—	—	—	—

Result of the statistical analysis of behavioral data in the Morris Water Maze employing a three-way Analysis of ANOVA, having groups as the between-subjects factor and session and trial within-subjects factors, followed by Bonferroni post-test. The level of significance considered as significant was set at 5% ($P < 0.05$).

the RJ group was observed. When comparing the groups STZ and STZ-RJ, a reduction of 40% in the STZ-RJ group was observed. In the hilus area, there was neither significant effect of the STZ Factor [F (3,15) = 1,755; p = 0.201] or RJ Factor [F (3.15) = 1.162; p = 0.2945] on the number of labeled nuclei. However, ANOVA showed a significant increasing oxidative effect of the STZ Factor [F (3,15) = 15,97; p = 0.001] and a significant decreasing oxidative effect of RJ Factor [F (3.15) = 4.713; p = 0.046] on the optical intensity of the labeling, indicating a decrease of the oxidative stress levels in the hilus region (Figure 13) after RJ consumption. A reduction of 61% on the intensity of the labeling in the RJ group (CTR x RJ) and 33.4% in the STZ-RJ group (STZ X STZ-RJ) were observed when compared to their respective controls. In the CA1 region, there was neither significant effect of the STZ Factor [F (3,15) = 3,226; p = 0.092] or RJ Factor [F (3.15) = 0.8332; p = 0.375] in the change in the number of labeling. In the same way, when the optical intensity of labeling was observed, there is neither significant effect of the STZ Factor [F (3,15) = 3,7; p = 0.075] or RJ treatment Factor [F (3,15) = 1,299; p = 0.2736] (Figure 13). In the CA3 region, there was neither significant effect of the STZ Factor [F (3.15) = 0.6827; p = 0.42] or RJ factor [F (3.15) = 0.8288; p = 0.3753] on the number of labeling nuclei. When the optical intensity was analyzed, there was neither significant effect of the STZ Factor [F (3.15) = 0.2826; p = 0.601] or RJ Factor [F (3.15) = 0.2016; p = 0.659] (Figure 13).

3.6. Neurogenesis

According to ANOVA, the STZ Factor had a significant effect on the reduction of the number of DCX-positive cells in DG [F (3,30) = 15.85; p < 0.001] and the RJ Factor showed a significant effect on the differentiation of new neurons in the DG [F (3,30) = 5,703; p = 0.023]. The STZ Factor had a significant effect on the reduction of the number of Brdu-positive neurons in DG [F (3,8) = 14,95; p = 0.004] and RJ Factor showed no significant effect on the survival rate of the new neurons in the DG [F (3,8) = 5.989; p = 0.413] (Figure 14).

3.7. Glycemic levels

No difference was observed in the glycemic levels among the groups. According to ANOVA, the STZ Factor [F (3,21) = 0,403; p = 0.532] and RJ factor [F (3,21) = 2.03; p = 0.168) did not have a significant effect on the glycemic levels of the animals (Figure 15).

3.8. Body weight

According to ANOVA, differences in weight measurements were observed among the groups [F (3,67) = 532.4; p < 0.001] at the time of the experimental period [F (3,67) = 75.29; p < 0.001] (Figure 15). The post-test indicates that the groups receiving icv-STZ injection (STZ and STZ-RJ groups) present a reduction of body weight in relation to the CTR group at day 14 (D14). On the other hand, the animals that died during the experimental period presented a notable reduction in body weight as measured on day 7 (D7) (Figure 16).

3.9. Mortality rate

Figure 17 shows the survival of the animals belonging to the groups CTR, RJ, STZ and STZ-RJ at the end of the test period, 21 days after icv-STZ injection surgery. The animals receiving icv injection of Ringer's

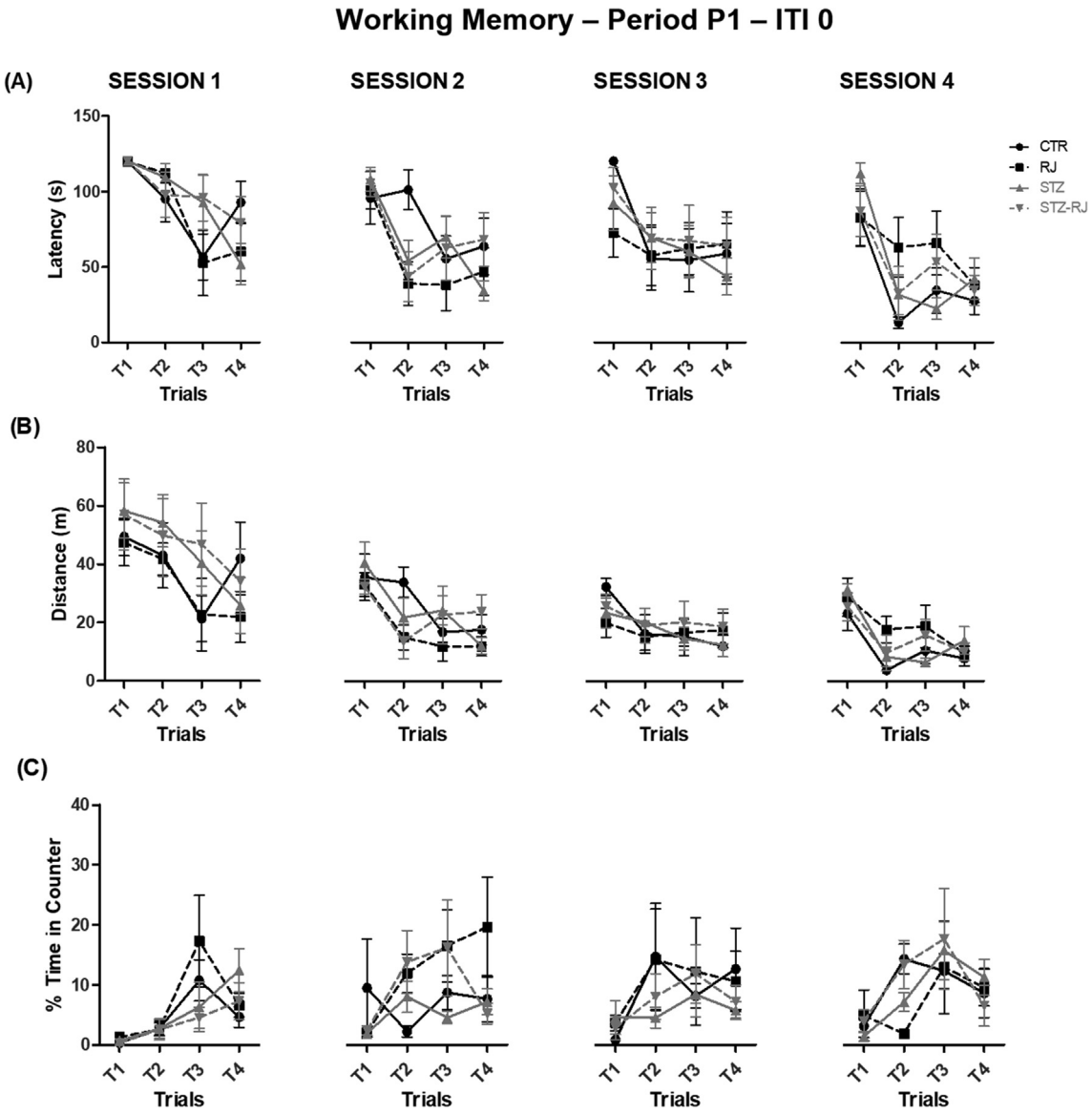

Figure 7. Performance of the animals throughout the sessions in terms of (A) Latency, (B) Distance and (C) % time within the critical counter (data expressed as mean ± standard error) in the Morris Water Maze Task along 4 Trials per session (the intertrial interval was "zero" – ITI0), and 4 Sessions (one Session per day). Groups (prospectively): CTR = Control (n = 6); RJ = Royal Jelly (n = 6); STZ = Streptozotocin (n = 7); STZ-RJ = Streptozotocin-Royal Jelly (n = 7).

solution (CTR and RJ groups) had a survival rate of 100%. It is observed according to ANOVA that the animals that received icv injection of STZ had a reduction of survival rate [$F(3,123) = 14{,}03$; $p < 0.001$]. The animals of the STZ group had a survival rate of 74% and the animal of the STZ-RJ group showed a survival rate of 79%.

4. Discussion

In this study we showed behavioral and histochemical changes induced by the intracerebroventricular injection of streptozotocin (icv-STZ). The performance impairment in the working memory of the animals submitted to the icv-STZ injection was evidenced throughout the observation period, from the 4th to the 20th day after injection. Histochemical studies demonstrated hippocampal neurodegeneration, increased generation of reactive oxygen species, and reduced proliferation and survival of new hippocampal neurons. In addition, we demonstrated the beneficial effects of royal jelly (RJ) long-term treatment that was able to attenuate the deleterious effects induced by the icv-STZ injection, as evidenced in behavioral tests, when the task of the working memory test in the Morris Water Maze was made with the intertrial interval (ITI) defined as zero. Molecular studies also showed that RJ long-term treatment in animals of the icv-STZ group produced a reduction in the extent of hippocampal neurodegeneration and in the generation of reactive oxygen species, in addition to an increase in the proliferation of new neurons. Thus, RJ showed beneficial effects for animals with cognitive impairment, neurodegeneration and high levels of oxidative stress. Moreover, the absence of significant changes in the swimming speed, in the locomotion and anxiety-like behavior of the animals of the control and icv-STZ groups is a consistent indication that they did not present a motor or motivational deficit.

The icv-STZ model has been accepted for presenting the main characteristics of AD, including cognitive impairment and therefore has been used for pathological studies and development of therapeutic proposals for SAD (Salkovic-Petrisic et al., 2006, 2013). We demonstrated that the icv-STZ injection was able to cause learning and working spatial memory impairments in rats. This deleterious effect can be observed from the first session, which was performed on the 4th day after icv-STZ injection and lasted throughout the observation period, that is, until the 20th

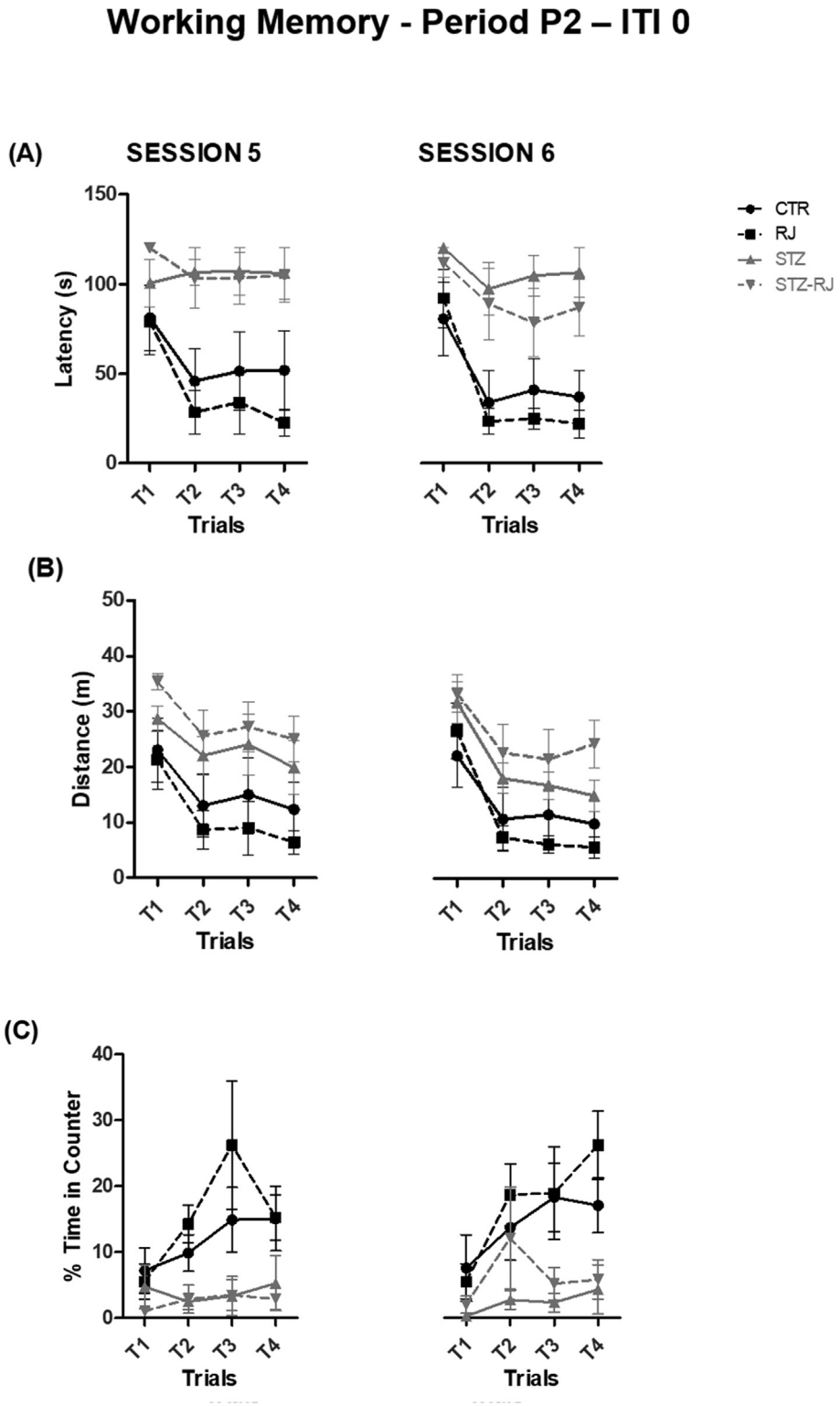

Figure 8. Performance of the animals throughout the sessions in terms of (A) Latency, (B) Distance and (C) % time within the critical counter (data expressed as mean ± standard error) in the Morris Water Maze Task along 4 Trials per session (the intertrial interval was "zero" – ITI0), and 2 Sessions (one Session per day). Groups: CTR = Control (n = 6); RJ = Royal Jelly (n = 6); STZ = Streptozotocin (n = 7); STZ-RJ = Streptozotocin-Royal Jelly (n = 7).

post-operative day. This loss was persistent in the acquisition and maintenance of spatial information in relation to the platform location, which is represented by the increase in latency and distance to find the platform in each of the trials within each session (day). Our data corroborate with previous studies (Santos et al., 2012; Zamani et al., 2012). We also verified that the animals that received icv-STZ injection showed a lower level of improvement in spatial memory performance along the 4 trials within each session of the Morris Water Maze test, which is characteristic of animals with hippocampal lesion, including the dentate gyrus, as shown by Xavier et al. (1999).

In the Morris Water Maze test it is also possible to evaluate the retention time of the working memory by the variation of the intertrial

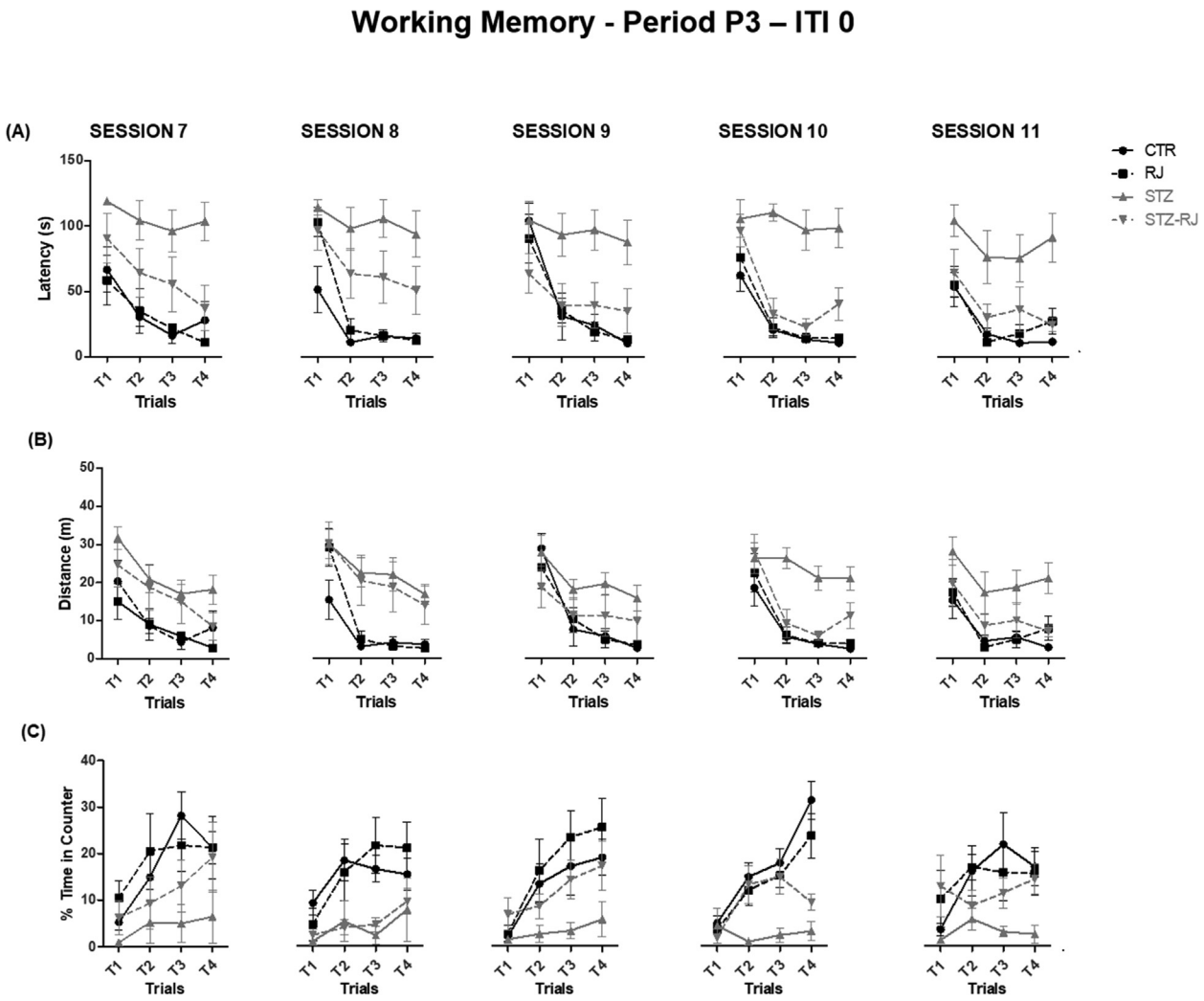

Figure 9. Performance of the animals throughout the sessions in terms of (A) Latency, (B) Distance and (C) % time within the critical counter (data expressed as mean ± standard error) in the Morris Water Maze Task along 4 Trials per session (the intertrial interval was "zero" – ITI0), and 5 Sessions (one Session per day). Groups: CTR = Control (n = 6); RJ = Royal Jelly (n = 6); STZ = Streptozotocin (n = 7); STZ-RJ = Streptozotocin-Royal Jelly (n = 7).

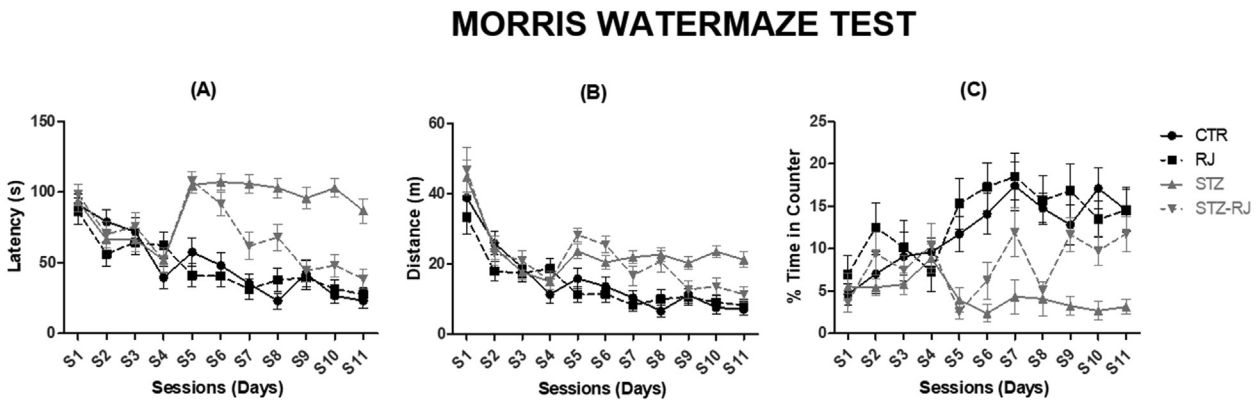

Figure 10. Performance of the animals throughout the sessions in terms of (A) Latency, (B) Distance and (C) % time within the critical counter in parameters in the Periods P1 (S1-S4), P2 (S5-S6) and P3 (S7-S11) (data expressed as mean ± standard error) in the Morris Water Maze Task along 4 Trials per session (the intertrial interval was 15 min - ITI15) and 11 Sessions (one Session per day). Groups: CTR = Control (n = 7); RJ = Royal Jelly (n = 8); STZ = Streptozotocin (n = 7); STZ-RJ = Streptozotocin-Royal Jelly (n = 9).

interval (ITI) (Olton, 1983; Santos, 1999). In our study we reduced the ITI from 15 min to zero in order to evaluate how much this reduction could lead to an improvement of the performance of the injured animals compared to that observed when the ITI was 15 min. With the application of this strategy we observed that the performance of the animals of the STZ group presented a reduction in the distance parameter in relation to the first trial (T1> T2 = T3 = T4), but without alteration in latency and percentage of time in the counter area.

Table 5. Working memory evaluated by Morris water maze test on the 4th and 6th day after the icv injection of STZ or control solution with an intertrial interval of 0 min (Period 2 - ITI 0).

Parameters	Group		Trial		Trial/Group	
	F 3,204	*P*	F 3,204	*P*	F 3,204	*P*
LAT	20,495	<0,001	9,806	<0,001	—	—
DIST	10,145	<0,001	19,104	<0,001	—	—
%T-A_CONT	7,103	0,002	7,381	<0,001	—	—

Parameters	Day		Day/Group		Day/Trial		Day/Trial/Group	
	F 3,204	*P*	F 3,204	*P*	F 3,204	*P*	F 3,204	*P*
LAT	—	—	—	—	—	—	—	—
DIST	—	—	—	—	—	—	—	—
%T-A_CONT	—	—	—	—	—	—	—	—

Result of the statistical analysis of behavioral data in the Morris Water Maze employing a three-way Analysis of ANOVA, having groups as the between-subjects factor and session and trial within-subjects factors, followed by Bonferroni post-test. The level of significance considered as significant was set at 5% ($P < 0.05$).

Table 6. Working memory evaluated by Morris water maze test along the RJ long-term administration in rats icv injected with STZ or control solution with an intertrial interval of 0 min (Period 3 - ITI 0).

Parameters	Group		Trial		Trial/Group	
	F 3,516	*P*	F 3,516	*P*	F 3,516	*P*
LAT	14,209	<0,001	65,537	<0,001	3,809	0,001
DIST	10,284	<0,001	72,31	<0,001	—	—
%T-A_CONT	8,833	0,001	28,511	<0,001	3,097	0,004

Parameters	Day		Day/Group		Day/Trial		Day/Trial/Group	
	F 3,516	*P*	F 3,516	*P*	F 3,516	*P*	F 3,516	*P*
LAT	4,056	0,005	—	—	—	—	—	—
DIST	—	—	—	—	—	—	—	—
%T-A_CONT	—	—	—	—	—	—	—	—

Result of the statistical analysis of behavioral data in the Morris Water Maze employing a three-way Analysis of ANOVA, having groups as the between-subjects factor and session and trial within-subjects factors, followed by Bonferroni post-test. The level of significance considered as significant was set at 5% ($P < 0.05$).

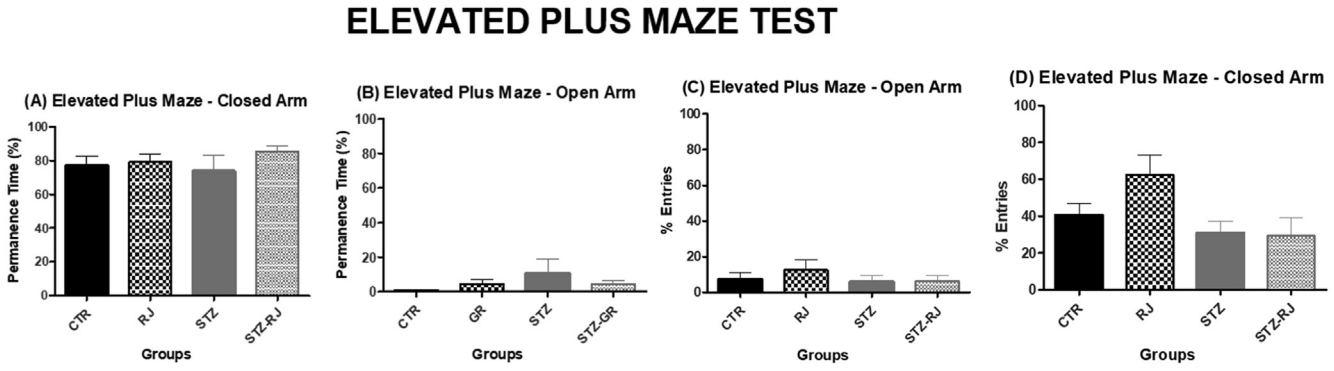

Figure 11. Effects of icv-STZ injection or physiological solution and oral administration of royal jelly or distilled water evaluated in the Elevated Plus Maze Test. (A) % Time - Closed Arm, (B) %Time - Open Arm, (C) %Entries - Open Arm, (D) %Entries - Closed Arm. CTR (n = 7); RJ (n = 8); STZ (n = 8); STZ-RJ (n = 9). CTR = Control; RJ = Royal Jelly; STZ = Streptozotocin; STZ-RJ = Streptozotocin-Royal Jelly. (A) Data expressed as mean ± standard error. For analysis of the data, the analysis of variance (Two-way ANOVA) was used, followed by the Bonferroni post-test. The level of significance was set at $p < 0.05$.

However, the association of two facilitating factors i.e., the reduction of the ITI to zero and RJ oral administration during 2 weeks in icv-STZ animals (STZ-RJ group) was able to cause beneficial effects on the working memory performance, which are represented by a significant decrease in latency, distance, and increase of percentage of time in the Counter area over the trials of each session. In addition, the reduction of the ITI to zero showed that the animals of the STZ-RJ group showed a gradual improvement in the search performance of the platform that tends to match the animals of the control groups (CTR and RJ) throughout the sessions (S7-S11), regarding the parameters latency, distance, and percentage of time in the Counter area. In this way, our results show that the working memory impairment of the animals of the STZ group was slightly attenuated when the ITI was zero but that animals injured by icv-STZ injection receiving oral treatment with RJ (STZ-RJ group) presented a significant improvement in working memory performance that gradually was accentuated along the RJ treatment, indicating a positive effect of RJ oral long-term treatment. Moreover, RJ positive effects have been demonstrated in rats submitted to the SAD model via icv-STZ injection (Zamani et al., 2012) and naturally aged rats chronically treated with RJ (Pyrzanowska et al., 2014).

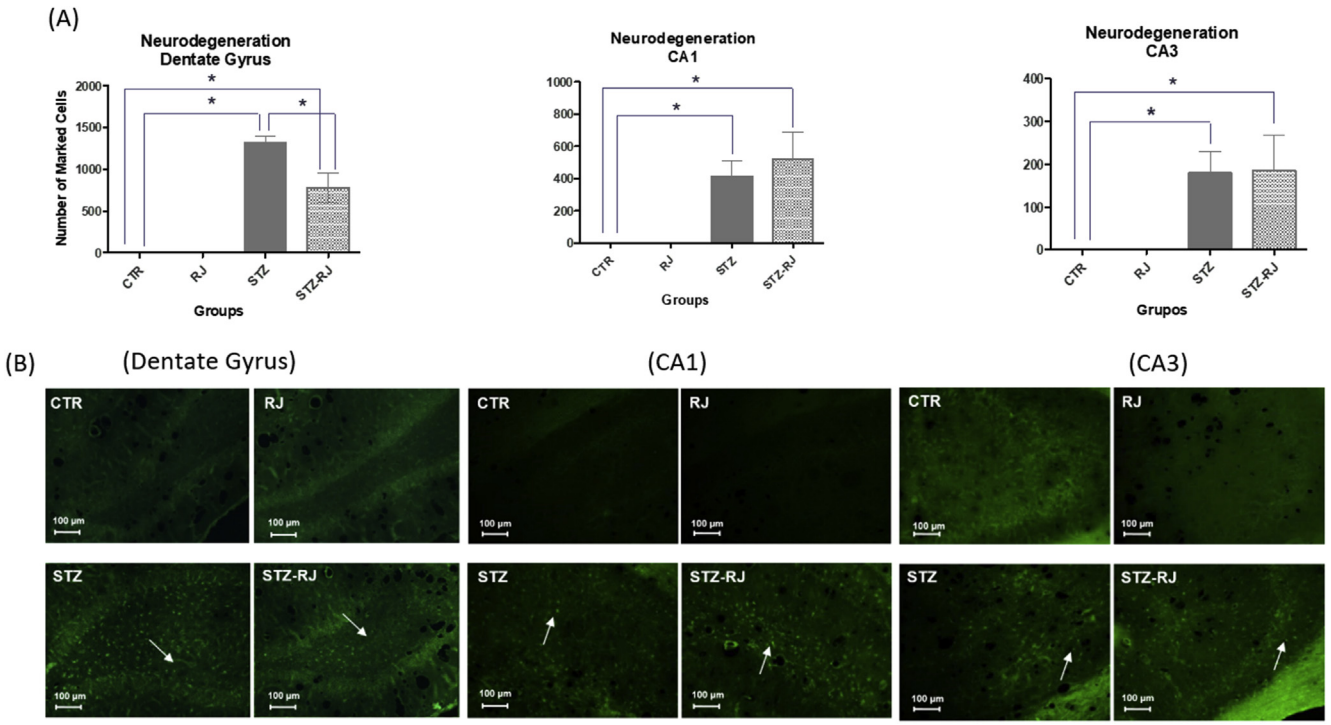

Figure 12. (A) Quantification of neurodegeneration (FJ-positive cells) in the hippocampus. (B) Digital images of coronal sections of the hippocampus showing the FJ (indicated by arrow) labeling 21 days after the injection of STZ (STZ and STZ-RJ) or physiological solution (CTR and RJ). CTR (n = 5); RJ (n = 5); STZ (n = 5); STZ-RJ (n = 5). CTR = Control; RJ = Royal Jelly; STZ = Streptozotocin; STZ-RJ = Streptozotocin-Royal Jelly. (A) Data expressed as mean ± standard error. For analysis of the data, the analysis of variance (two-way ANOVA) was used, followed by the Bonferroni post-test. The level of significance was set at $p < 0.05$.

In the present work we chose to evaluate spatial working memory due to the knowledge that working memory impairments are present in the early stages of Alzheimer's disease (Bird et al., 2010). A study aimed at understanding the core of cognitive processes that support topographic disorientation presented by patients in the initial stage of AD has shown that these patients are particularly sensitive to short-term memory deficit when submitted to a spatial memory test for topographic representation of a landscape (Bird et al., 2010). These findings reinforce the relevance of investigating working spatial memory in animal models of AD. In this regard, Xavier et al. (1999) showed the importance of using the Morris water maze to investigate this type of memory. Thus, the oral treatment with RJ provided a positive effect in the reduction of the injuries caused by the icv-STZ injection in the tests with ITI = zero, but these effects were not observed in the tests with ITI = 15min. In this way, we can say that RJ acts to bring benefits to retention mechanisms of spatial memory over a period. Prolonged ITI, as 15min, causes the loss of information previously held by the animals in the STZ-RJ group.

Santos et al. (2012) demonstrated that animals submitted to the icv-STZ model present neurodegeneration in the hippocampus between 1 and 15 days after icv-STZ injection, but no more FJ-positive cells are found after 30 days post-icv-STZ injection. Our work corroborates with these data since we also identified the presence of neurodegeneration in the DG, CA1 and CA3 hippocampal areas, evaluated 21 days after the icv-STZ injection. Several studies have shown that hippocampal lesions disrupt spatial orientation and indicate that the integrity of hippocampal formation is necessary to create a cognitive map that supports flexible spatial navigation based on a reference strategy of the place (Xavier and Costa, 2009). Thus, in our study, we could associate neuronal death in the hippocampal region of STZ-injured animals with reduced spatial memory performance of the animals during the water maze test. In addition, our study showed for the first time that RJ oral administration for 14 days, started 7 days after the icv-STZ injection, reduces neuronal degeneration in the hippocampal dentate gyrus, indicating a neuroprotective effect, which it was also evidenced by the improvement of spatial working memory performance of these animals in the Morris Water Maze test.

A growing body of evidences indicates that the administration of icv-STZ generates reactive oxygen species, a type of free radical, which results in increased oxidative stress (Shoham et al., 2007). In our work, we evaluated the oxidative stress by the levels of the superoxide anion ($O2 \bullet -$), represented by the dihydroethidium oxidation (DHE) labeling. The oxidation of DHE in a fluorescent product estimates the levels of intracellular reactive oxygen species (Bindokas et al., 1996; Liberman et al., 2008). DHE, a reduced form of the ethidium, enters freely in the cell and reacts rapidly with $O2 \bullet -$ forming ethidium. When oxidized to ethidium, it binds to the DNA causing the amplification of the red fluorescence. The oxidation of DHE is quantitatively proportional to the concentration of $O2 \bullet -$ in the cell (Sharikabad et al., 2001).

We identified that animals receiving icv-STZ injection (STZ and STZ-RJ) had a higher oxidative stress index observed by the quantity of labeling related to the oxidative stress levels in the DG region of the hippocampus. In addition, the animals injured by STZ showed a significant increase on the intensity of the labeling in the DG and hilus, corroborating with the theory of the oxidative effect of the icv-STZ injection. Our results also showed that the icv-STZ injection did not cause a significant increase in the generation of reactive oxygen species in the hippocampal CA1 and CA3 regions. This may indicate that the STZ injection into the lateral ventricles did not able to reach these regions or that they are not sensitive to this compound. Regarding the anatomical location of the DG and hilus they are closer to the lateral ventricle than CA1 and CA3 regions. Moreover, DG and hilus regions present a reverberating circuitry very susceptible to deleterious effects (Amaral et al., 2007). The effect of oxidative stress in rodents submitted to the icv-STZ model has been widely reported by several groups (Deshmukh et al., 2009; Mehan et al., 2011). Researches indicate that treatment with antioxidants may alleviate STZ-induced cognitive impairment in the brain (Weinstock and Shoham, 2004; Ishrat et al., 2009a,b; Dhull et al., 2012; Javed et al., 2012). One of the recognized pharmacological properties present by RJ is

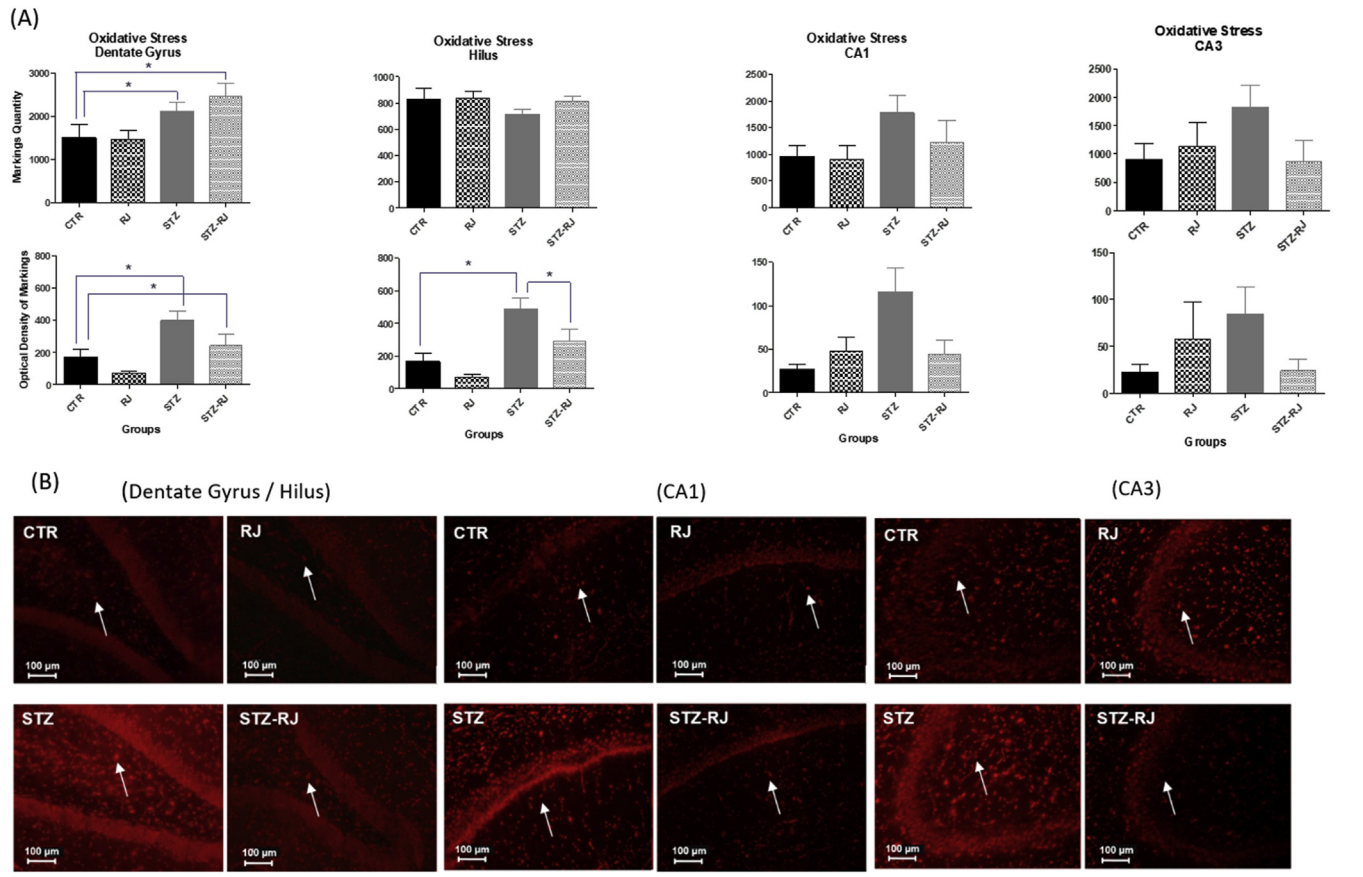

Figure 13. (A) Number of labelings of reactive oxygen species and optical intensity of the labeling of the reactive oxygen species (DHE oxidation levels) in the hippocampus. (B) Digital images of coronal sections of hippocampus, showing the DHE labeling 21 days after the injection of STZ (STZ and STZ-RJ) or physiological solution (CTR and RJ) (arrows indicate a sample of the label). CTR (n = 4); RJ (n = 4); STZ (n = 5); STZ-TJ (n = 6). CTR = Control; RJ = Royal Jelly; STZ = Streptozotocin; STZ-RJ = Streptozotocin - Royal Jelly. Data expressed as mean ± standard error. For analysis of the data, the analysis of variance (two-way ANOVA) was used, followed by the Bonferroni post-test. The level of significance was set at $p < 0.05$.

the capacity to eliminate free radicals, indicating that RJ is a very effective antioxidant (Pavel et al., 2011). The antioxidant effects of RJ have been demonstrated in different organ and cell types, including inhibition of lipid peroxidation, both in vitro and in vivo (El-Nekeety et al., 2007; Guo et al., 2008; Zahmatkesh et al., 2014). In this respect, we observed for the first time that RJ long-term oral treatment showed an antioxidant effect in the hilus region and a tendency of antioxidant effect in the region of the DG by reducing the level of intensity of the labels related to the superoxide anion index (O2 -). Moreover, the hippocampus, where it was identified the remarkable increase of oxidative stress, was also the region where we found a significant index of neurodegeneration induced by the icv-STZ injection. In this way, we can consider that the oxidative stress induced by icv-STZ injection, at least in part, is responsible for cell damage and consequent neurodegeneration in the DG of the hippocampus, and that the antioxidant effect of RJ may have contributed for the reduction of neuronal death rates in this region. Corroborating with our study, recently a study using a rabbit model of Alzheimer's disease showed that RJ long-term oral administration ameliorate amyloid deposition, reduced the neuronal loss and enhanced anti-oxidative capacities in AD rabbits cortex and hippocampus brain areas (Pan et al., 2018).

Considering the importance of the hippocampal formation for the spatial memory processing, and that the DG is the main final region of the perforant pathway, it is reasonable to consider that DG acts in the first stage of information processing that leads to the production of memories (Amaral et al., 2007). In addition, the DG peculiar neuroanatomy predicts that it performs a specific task of information processing that it receives from the entorhinal cortex and finally transmits it to the CA3 region of the hippocampus (Amaral et al., 2007). Regarding the neurodegenerative effects in the hilar region induced by STZ and the neuroprotective effects provided by the RJ oral treatment, the studies indicate the existence of an important modulatory circuitry, which has significant influence in the memory processing in DG. This area contains several GABAergic inhibitory neurons within the hilus and in the molecular layer and, in addition, the polymorphic layer has a wide variety of cell types (Amaral et al., 2007). The main hilus cells are glutamatergic mossy cells, which receive afferents from the granular cells via mossy fibers, and then innervate local interneurons, as well as deliver feedback projections to the granular cells (Amaral et al., 2007; Christian et al., 2014). In this regard, we consider that the royal jelly neuroprotective effects observed in the DG region, concerning the oxidative stress and neurodegeneration reduction, could be the responsible for the preservation of the hippocampal circuitry, especially in the DG. Furthermore, this vision collaborates with the maintenance and/or partial recovery of information processing mechanisms for the formation of memories; preservation is observed both in the working memory, demonstrated in the present work, and previously by other researchers in the reference memory (Zamani et al., 2012).

Studies have been carried out to understand the role of neurogenesis in hippocampal functioning (Deng et al., 2010; Aimone and Gage, 2011; Lazarini and Lledo, 2011; Sahay et al., 2011). Several conditions that decrease neurogenesis in the DG of rodents are associated with learning deficits such as stress, increased glucocorticoid levels and aging (Montaron et al., 2006; Drapeau et al., 2007). Similarly, conditions that increase neurogenesis, such as environmental enrichment and physical activity, tend to increase the performance of hippocampal-dependent

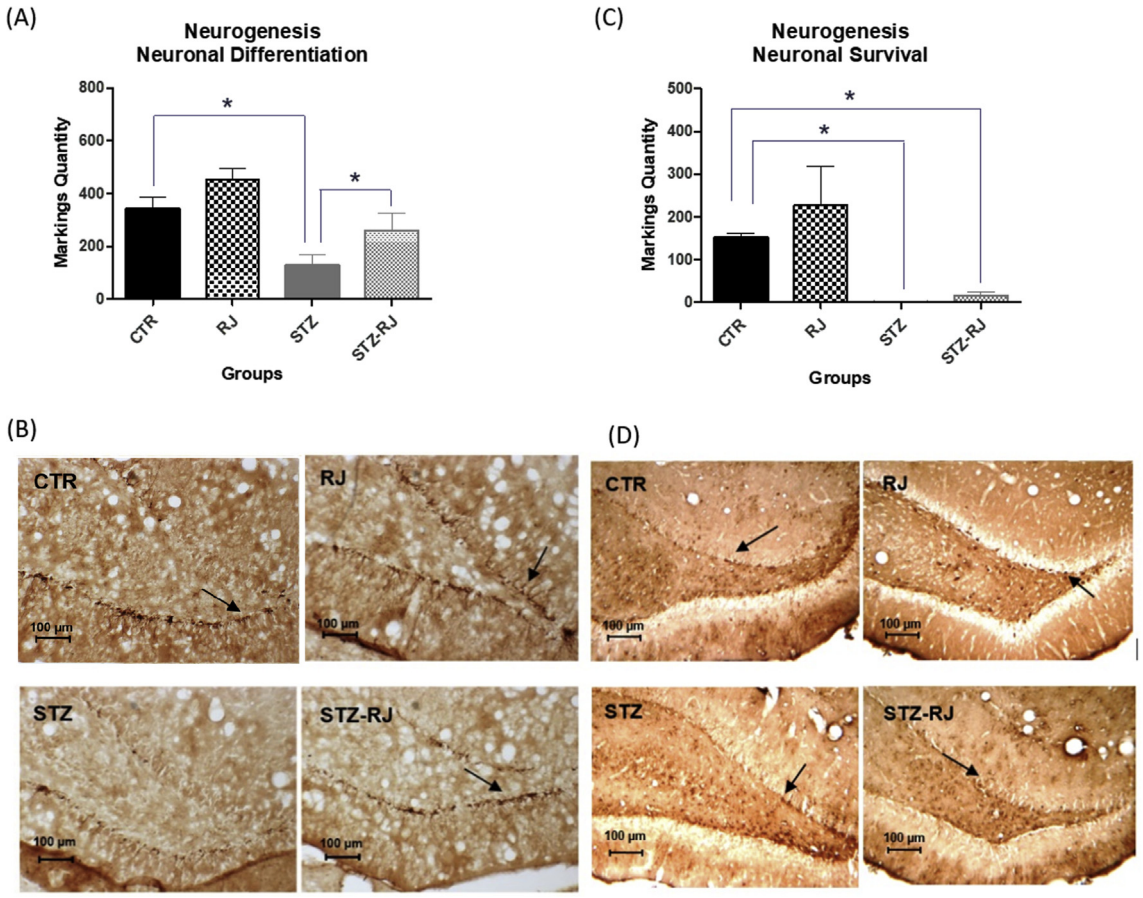

Figure 14. (A) Quantification of proliferation and neuronal differentiation (DCX-positive cells) in the dentate gyrus of the hippocampus. (B) Digital images of coronal sections of hippocampus, illustrating the DCX labeling 21 days after the injection of STZ (STZ and STZ-RJ) or physiological solution (CTR and RJ) (the arrows indicate a sample of the label). CTR (n = 7); RJ (n = 9); STZ (n = 8); STZ-RJ (n = 10). (C) Quantification of neuronal survival (Brdu-positive cells) in the dentate gyrus of the hippocampus. (D) Digital images of coronal sections of hippocampus, illustrating Brdu labeling 21 days after Brdu injection (35 days after icv injection). CTR (n = 3); RJ (n = 3); STZ (n = 3); STZ-RJ (n = 3). CTR = Control; RJ = Royal Jelly; STZ = Streptozotocin; STZ-RJ = Streptozotocin-Royal Jelly. Data expressed as mean ± standard error. For analysis of the data, the analysis of variance (two-way ANOVA) was used, followed by the Bonferroni post-test. The level of significance was set at $p < 0.05$.

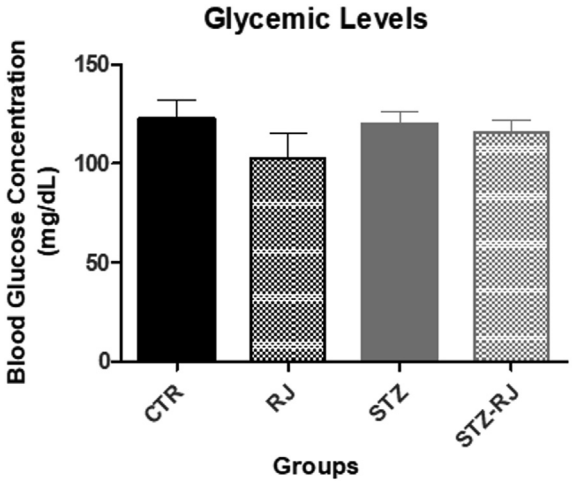

Figure 15. Effects of intracerebroventricular injection of streptozotocin and/or oral treatment with royal jelly on glycemia in rats. CTR (n = 4); RJ (n = 4); STZ (n = 8); STZ-RJ (n = 9). CTR = Control; RJ = Royal Jelly; STZ = Streptozotocin; STZ-RJ = Streptozotocin-Royal Jelly. Data expressed as mean ± standard error. For analysis of the data, the analysis of variance (two-way ANOVA) was used, followed by the Bonferroni post-test. The level of significance was set at $p < 0.05$.

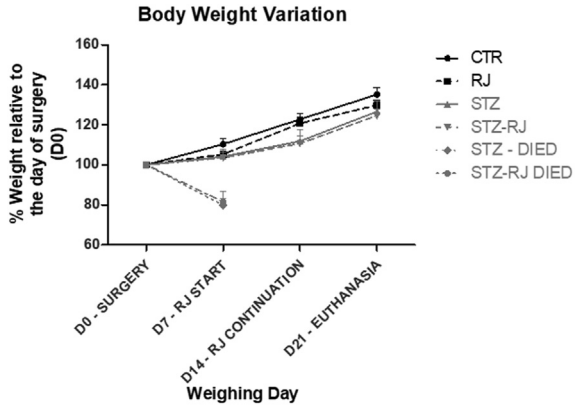

Figure 16. Effects of intracerebroventricular injection of streptozotocin and oral treatment with royal jelly on the body weight of rats. CTR (n = 10); RJ (n = 13); STZ (n = 11); STZ-RJ (n = 15); STZ-DIED (n = 11); STZ-RJ-DIED (n = 7). CTR = Control; RJ = Royal Jelly; STZ = Streptozotocin; STZ-RJ = Streptozotocin-Royal Jelly. Values represented by the percentage of weight measured on the day of surgery icv (D0). Data expressed as mean ± standard error. For analysis of the data, the analysis of variance (two-way ANOVA) was used, followed by the Bonferroni post-test. The level of significance was set at $p < 0.05$.

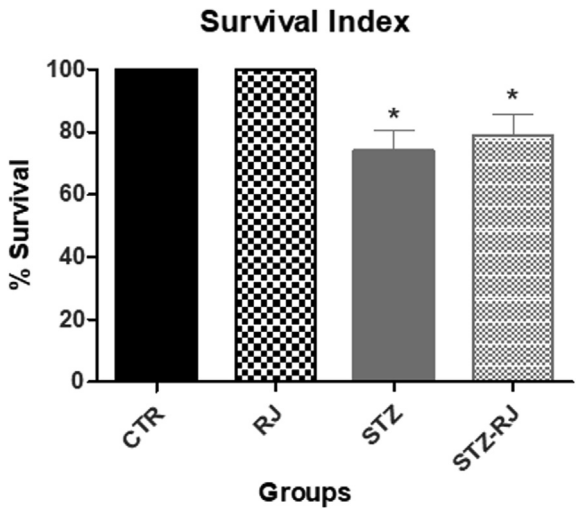

Figure 17. Effects of intracerebroventricular injection of streptozotocin and oral treatment with royal jelly on survival index of rats. CTR (n = 22); RJ (n = 25); STZ (n = 42); STZ-RJ (n = 38). CTR = Control; RJ = Royal Jelly; STZ = Streptozotocin; STZ-RJ = Streptozotocin-Royal Jelly. Values represented by the percentage of live rats until the end of the experimental period. Data expressed as mean ± standard error. For analysis of the data, the analysis of variance (two-way ANOVA) was used, followed by the Bonferroni post-test. * The level of significance was set at $p < 0.05$ in relation to the CTR.

tasks (Kempermann et al., 1997; Van Praag et al., 1999, 2005). However, due to the complex interaction factors regulating adult neurogenesis, a possible correlation between the number of new neurons generated and learning performance is not yet understood in a direct cause and effect.

Moreover, recent studies propose that adult neurogenesis should not be characterized as a cellular replacement process, but rather as a mechanism that maintains the plasticity of the hippocampal circuit by the continuous addition of immature neurons and new neurons endowed with unique properties, in addition to the structural plasticity promoted by integration of these new neurons into the existing circuitry. The doublecortin (DCX) labeling method allows quantifying the existence of immature neurons, that is, neuroblasts that have not yet integrated into the neuronal circuitry. A recent study also showed that the icv-STZ injection causes a reduction in the generation of new neurons in the septal hippocampal region after 3 months of icv-STZ injection, but in this study the reduction of neurogenesis had not been observed after 1 month of the icv-STZ injection (Sun et al., 2015). In contrast, studies by Qu et al. (2012) identified reduction of progenitor cell proliferation and differentiation of new neurons in the rat dentate gyrus region 21 days after icv-STZ injection. Our study also shows that the icv-STZ injection is able to reduce neurogenesis, when we quantify the level of immature neurons (number of DCX-positive cells) in the region of the dentate gyrus 21 days after the STZ-induced lesion. The literature shows that there is a controversy about the relation between neurogenesis and the learning and memory processes (Leuner et al., 2007) and about the influence of neurogenesis in the pathology of AD (Boekhoorn et al., 2006; Herring et al., 2009). In the present work it was evaluated the effect of RJ long-term oral administration on differentiation of new neurons in vivo showing for the first time that RJ was able to raise the level of proliferation of new granular neurons in the DG of rats. Moreover, the analysis of the survival of the new granular neurons in DG indicated that the icv-STZ injection significantly reduced the number of Brdu-positive neurons but that the oral treatment with RJ showed no changes in the amount of new mature neurons integrated into the cell layer granular.

Based on our results, we could suggest that icv-STZ reduced neurogenesis and royal jelly increased neuronal proliferation in the DG are related, at least in part, to the memory loss of the animals of the STZ group and the improvement performance of the animals of the STZ-RJ group, respectively. In addition, a growing number of studies have shown that the dysfunction of adult hippocampal neurogenesis may play a causal role in several brain disorders (Parent, 2003; Sahay and Hen, 2007; Kempermann et al., 2008; Winner et al., 2011). Therefore, studies on adult neurogenesis may promote the development of potential new therapeutic strategies for brain disorders. In addition, the basic principles learned with the neuronal development and synaptic integration of the newborn neurons in the process of neurogenesis may also provide valuable information for the future development of cellular replacement therapy in the nervous system. Thus, the proliferation augment of new neurons provided by the oral treatment with RJ can also be considered as a result of considerable neuroprotective value. In this way, studies indicate that the newly generated, still immature neurons, exert great influence on the dynamics of the hippocampal circuitry as well as impact on cognitive and behavioral aspects related to learning and memory, pattern recognition, stress and mood disorders (Christian et al., 2014). Concerning the neuroprotective effect induced by RJ identified in this work by the increase in the number of proliferating new neurons in the hippocampal DG, we could hypothesize that its plays an important role in the recovery of memory performance, acting together with the others beneficial effects, reduction of the levels of oxidative stress and the neuronal degeneration.

Thus, all parameters analyzed in the present work are related to hippocampal neuroprotective effects of the royal jelly oral administration. These beneficial brain effects might be assigned to some RJ compounds that can be absorbed by gastro-intestinal tract and are able to pass through hematoencephalic barrier. In this regard, among the active substances in the RJ we highlight small peptides (obtained from the hydrolysis of major royal jelly proteins, MRJPs), free amino acids, the 10-carbon atoms fatty acids, 10-hydroxy-2-decenoic acid (10-HDA) and 10-hydroxydecanoic acid, besides AMP-N1 oxide (Cornara et al., 2017; Kocot et al., 2018). It has been shown that small peptides, with 2–4 amino acids residues isolated from RJ containing Tyr residues at C-terminal present strong hydroxyl-radical scavenging activity that is suggested act as free-radical scavengers (Guo et al., 2009). Concerning the 10-hydroxy-2-decenoic acid (10-HDA), the main and unique fatty acid found specifically in RJ, was demonstrated that it participates the production of important molecules for brain function as brain-derivate neurotrophic factor (BDNF) (Ito et al., 2003; You et al., 2018) and neurogenesis in neural progenitor cells (Hattori et al., 2007). Weiser et al. (2017) reported that 10-hydroxy-2-decenoic acid increased the viability and growth of primary hippocampal neurons and using in vitro models of age-related neurodegeneration showed that this fatty acid was able to reduce cell death. Moreover, a decreased anxiety in aged male rats treated with 10-HDA was observed (Weiser et al., 2017). Another RJ component that could respond, at least in part, for the neuronal benefits of RJ is the adenosine monophosphate (AMP) N1-oxide, which is found only in RJ, and present a neurotrophic factor. Thus, AMP N1-oxide induces neurite process, suppressed PC12 cell proliferation and stimulated expression of a specific protein of mature neurons, demonstrating its stimulatory activity to induce neuronal differentiation of PC12 cells. In addition, it has been suggested that AMP N1-oxide acts by adenylyl cyclase-coupled adenosine receptors and could play a role in modulating neuronal function via adenosine receptors (Hattori et al., 2007, 2010).

Taken together, the data obtained in this study demonstrate that the prolonged treatment with RJ reduced the deleterious effects on cognition, neurodegeneration and oxidative stress in animals submitted to the icv-STZ injection model. Thus, RJ presents a potential therapeutic value for the treatment of cognitive deficits and a beneficial action in neurodegenerative processes.

Declarations

Author contribution statement

T. Guardia: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

M. de Paulo: Performed the experiments;

J. Silva and A. Alves: Performed the experiments; Analyzed and interpreted the data.

L. Britto and G. Xavier: Conceived and designed the experiments; Analyzed and interpreted the data.

M. Sandoval: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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