Intracerebroventricular streptozotocin induces behavioral impairments and increases short-term C3 gene expression in the hippocampus of Wistar rats

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A non-transgenic rat model based on intracerebroventricular injection of streptozotocin (STZ) has been used as an animal model to investigate mechanisms associated to the late onset of sporadic Alzheimer's disease, such as anatomical and behavioral impairments. However, molecular aspects related to gene expression, mainly in the hippocampus, require more investigation. Thus, this study evaluated the early and late cognitive functions and hippocampal gene expression after STZ administration. Male Wistar rats were divided into 4 groups: STZ (injected bilaterally), control group for the early memory function evaluation (1 month after surgery = phase 1, same volume of vehicle), and the same treatment for the late memory function evaluation (4 months after surgery = phase 2). The animals were observed in the elevated plus maze to assess behaviors related to anxiety, risk-assessment and fear-related memories. The behavioral tests were followed by brain removal and hippocampal dissection for RNA extraction and qRT-PCR to assess the expression levels of 4 Alzheimer's disease related genes: Mapt, Apoe, C3 and Ps-1. Animals from both phases showed increased time percentage and number of entries into the open arms, indicating risk behavior associated with anxiety, and an increased time percentage in the center square for both exposures (re-test) when compared to the control group, suggesting working memory impairment related to an aversive event. Statistical analyses indicated that the STZ group presented alterations in anxiety, memory and risk assessment responses. Additionally, one month after STZ administration, C3 gene assays revealed an increased expression. Therefore, current data indicate that neuroinflammatory events linked to the expression of pro-inflammatory cytokines such as C3 are related to memory, anxiety and decision-making alterations.

Key words: Alzheimer's disease, neuroinflammation, anxiety, risk assessment

INTRODUCTION

Alzheimer’s disease (AD) is a progressive neurodegenerative disease which is clinically characterized by dementia and neurobehavioral impairments mainly on memory functions (Kar et al., 2004). Additionally, significant behavioral changes such as progressive memory loss, anxiety, agitation and irritability are observed.

AD is categorized into two types: late-onset sporadic AD and early-onset familial AD (Dorszewska et al., 2016). The first one represents 95% of all the cases (Bartolotti and Lazarov, 2016). Sporadic AD is a multifactorial disease caused by genetic, epigenetic, environmental and metabolic aspects (Iqbal and Grundke-Iqbal, 2005) such as decreased glucose metabolism and energy consumption (Heiss et al., 1991; Gong et al. 2006).

The primary pathological characteristics of AD are neurofibrillar tangles, intraneuronal lesions composed of an aggregation of hyper phosphorylated TAU protein and amyloid deposits, constituted of accumulated Aβ peptide (Goedert et al., 1991).

Studies have shown that ε4 allele of apolipoprotein E (APOE) gene is a key component related to genetic risk factor for AD (Pericak-Vance et al., 1991; Liu et al., 2013). The complete absence of fibrillar Aβ in
Apoe-null AD transgenic mice (Holtzman et al., 2000) suggested that this gene may be a key participant in Aβ fibrillization.

Moreover, mutations in presenilin-1 (PS-1) gene also lead to amyloid deposits in the mouse brain (Duff et al., 1996). Another typical pathological characteristic of AD is the presence of neurofibrillary tangles (Nelson et al., 2009). They are aggregates of MAPT-encoded microtubule-associated protein TAU that is directly involved in the organization, stabilization, and dynamics of microtubules (Weingarten et al., 1975; Cuchillo-Ibanez et al., 2008; Dubey et al., 2008).

Activation of microglia, which leads to higher levels of inflammatory proteins expression in the brain (Xiang et al., 2006), have been identified as biomarkers of AD genesis. Complement factor C3 is a central component of the complement system and a key inflammatory protein activated in Alzheimer’s disease (Maier et al., 2008).

Transgenic mouse is frequently used as a model to mimic familial AD symptoms and molecular aspects (Elder et al., 2010). Aiming to study the mechanisms associated with sporadic AD, an intracerebroventricular injection of streptozocin (icv-STZ) in rats has been consolidated as an animal model (Grünblatt et al., 2007; Grieb, 2016; Lester-Coll et al., 2006). STZ, a glucosamine-nitrosourea compound, is a DNA alkylating agent, which enters the cells exclusively via GLUT2 glucose transport protein, leading to death of insulin-producing cells (Kamat, 2015). Thereby there is a chronically decrease of cerebral glucose uptake which produces multiple effects similar to molecular, pathological, and behavioral features of Alzheimer’s disease (Grieb, 2016).

Studies have already described cognitive alterations, such as deficits in spatial memory 40 days after icv-STZ (Shoham et al., 2003). Additionally, 3 h after STZ injections, disruption of working memory has been observed followed by degenerative processes in the hippocampus at 1 and 15 days after STZ injections. Furthermore, memory disruptions increase over time (Santos et al., 2012) as demonstrated by mice that received icv-STZ and showed impairment in learning and spatial memory 21 days after STZ administration (Chen et al., 2012). Bao and coworkers (2017) also found that icv-STZ remarkably induced impairment on learning and memory functions, besides loss of dendritic and synaptic plasticity in rats, 37 days after icv-STZ. Gene expression analysis in this animal model has been performed as an attempt to elucidate molecular aspects of the disease (Grünblatt et al., 2004; Hosseinzadeh et al., 2015; Gupta et al., 2018).

In spite of those behavioral experiments performed in icv-STZ model, more studies related to behavior and gene expression in AD genesis in a broader period are essential for the comprehension of the disease progression. Differences in gene expression associated with behavioral aspects in icv-STZ rat model can lead to new perspectives regarding prevention and treatment of sporadic AD, the most prevalent type observed in the population. Therefore, the aim of this study was to investigate a possible relationship between neurobehavioral impairments and gene expression of Mapt, Apoe, C3 and Ps-1 in the hippocampus of icv-STZ rat model in two phases; early (1 month after icv-STZ) and late (4 months after icv-STZ) expression.

**METHODS**

Thirty-two male Wistar rats at the age of 90 days, weighing in average 350 g, were used in this study. The animals were obtained from the animal facility at the Universidade Positivo and remained under controlled conditions including light/dark cycle of 12 h (07:00 am to 07:00 pm) and temperature of 21°C with water and food ad libitum.

The animals were divided into four groups of eight rats each (n=8 per group) and housed into subgroups of four animals in opaque standard laboratory cages. Two groups were used as a control, in which icv injections of citrate buffer were administered (control group) and the other two groups received streptozotocin via icv (STZ group). One control and one STZ group were submitted to the elevated plus maze (EPM) and had their brains removed one month after the injections (phase 1). Four months later, the other two groups were submitted to the same procedure to evaluate short and long-term effects of STZ, respectively (phase 2, Fig. 1).

The experiments were carried out in accordance with the guidelines of the Brazilian College for Animal Experimentation and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All the procedures involving animal experimentation were approved by the Ethics Committee on Animal Use in Research of Universidade Positivo under protocol number 322.

Rats were anesthetized with a combination of ketamine (90 mg/kg) and xylazine (12 mg/kg) followed by their positioning in a stereotaxic frame.

A sagittal incision in the midline was performed until bregma became clearly visible. Based on Paxinos and Watson (2013), the stereotaxic coordinates used for the application of STZ (Sigma Aldrich) or 0.05 M Citrate Buffer pH 4.5 (solution of citric acid, Merck and sodium phosphate, Merck) bilaterally into the lateral ventricles from bregma were: -0.96 mm in the
anteroposterior axis, 1.8 mm in the medial-lateral axis and 3.6 mm in the dorsal-ventral axis.

Sixteen rats were submitted to bilateral injections of STZ (3 mg/kg) freshly dissolved in citrate buffer to assess the effects of icv-STZ injection (Salkovic-Petrisic et al., 2011; Santos et al., 2012). The alterations observed after this dose administration induce damage in essential structures for learning and spatial memory (Shoham et al., 2003), but do not cause undesired systemic effects. The same vehicle at the same stereotaxic coordinates was injected for the control group. A volume of 5 μL was injected in each hemisphere. Bilateral injections ensure homogeneous drug dispersion in both hemispheres, generating no laterality-dependent effects.

Elevated plus maze

Anxiety, risk assessment and aversive episodic memory were evaluated through EPM tasks. Episodic memory was evaluated by the test-retest paradigm, which consists of repeated exposures to the EPM in a pre-established inter-trial-interval (Sharma and Kulkarni, 1992; Tanyeri, 2014; Mutlu et al., 2015). Anxiety was assessed using the time percentage and absolute number of entries in the open arms of the apparatus (Lamprea et al., 2000; Carobrez et al., 2001; Rasmussen et al., 2001) and risk assessment related behaviors was evaluated using time percentage spent in the center square (Rodgers et al., 1997).

The EPM presented two opposite facing open arms (50 × 10 × 0.5 cm), two closed arms (50 × 10 × 10 cm) and a center square (4 cm long × 4 cm wide) forming a plus shape. Each rat was placed in the center square facing a closed arm, under constant and indirect light for 5 min. After this procedure, the maze was cleaned with alcohol 70% and a retest was conducted with inter-trial-interval (ITI) of 5 min. A retest 5 min after the first exposure has shown that rats are able to use working memory, a well-known hippocampal dependent cognitive function, to acquire and remember about the aversive memory represented by the exposure to an open and elevated environment such as the EPM in the first exposure (Da Silva et al., 2019).

Behavioral patterns were measured from the time each animal performed the movements, using Pluz-MZ v1.1 Software. During all the evaluation period, groups were randomly tested and the investigator was unaware to the experimental group’s composition.
RNA extraction

Five animals from each group were submitted to RNA extraction and gene expression analyses. Animals were euthanized through deep isoflurane inhalation. After brain removal, hippocampus bilateral dissection was performed using a stereomicroscope. Then, the tissue was placed into a microtubule containing RNAlater™ Stabilization Solution (Thermo Fisher Scientific) for the RNA stabilization and protection. Hippocampal samples were stored at -20°C. After this procedure, a complete disruption of tissue structure and cell membranes was performed to release all the RNA contained in the sample using Trizol™ Reagent (Thermo Fisher Scientific).

PureLink™ RNA Mini Kit (Thermo Fisher Scientific) was used for total RNA extraction. Furthermore, TURBO™ DNase (Thermo Fisher Scientific) was added to eradicate DNA contamination from the total RNA preparations. The RNA purity, integrity and quantification were determined by NanoDrop® 200UV-Vis Spectrophotometer (NanoDrop Technologies), 1.5% agarose gel electrophoresis (supplementary Fig. 1A) and Quantus™ Fluorometer (Promega), respectively.

Reverse transcriptase reaction

One µg of total RNA was reverse-transcribed by High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) to generate cDNA. Reactions were performed under RNase-free conditions. These procedures consisted in adding 2 µL 10x RT Buffer, 0.8 µL 25x dNTP Mix, 2 µL 10x RT Random Primers, 1 µL MultiScribe Reverse Transcriptase 50U/µL, 4.2 µL nuclease free water, and 10 µL RNA (100 ng/µL) totaling 20 µL of reaction volume. Samples were incubated at 25°C for 10 min, 37°C for 2 h and 85°C for 5 min to inactivate reverse transcriptase in a Mastercycler personal (Eppendorf). Reverse transcription without enzyme was also performed as a negative control to assess possible gDNA contamination.

Primer design

Primer pairs for Apoe, Mapt and C3 were designed using Primer Express Software v.3.0.1 (Thermo Fisher Scientific). Gene sequences were obtained from Ensembl database (http://useast.ensembl.org/) which was also used to examine the genomic structure of genes and their transcripts. Primer sequence for Ps-1 was obtained following previous studies (Ricceri et al., 2004) and β-actin was selected as reference gene for gene expression analysis (Yamada et al., 2005; Soria-Fregoso et al., 2008; Hatami et al., 2010; Blanco et al., 2012). Primers covered at least two exons or were designed to amplify exons separated by large introns to avoid false-positive amplification of contaminating genomic DNA in the experimental samples.

All primer sequences were analyzed using BLAST tool to confirm the specificity to the target gene. The results showed absence of multi-locus matching individual primer sites. Polymerase chain reaction (PCR) and electrophoresis were performed to confirm the fragment size (supplementary Fig. 1B). Melting curve analysis was also performed to evaluate primer specificity (supplementary Fig. 2). Table I presents data and characteristics of each primer pair.

qRT-PCR analyses

The first-strand cDNA was used for qRT-PCR using RT² SYBR® Green qPCR Mastermix (Qiagen). Reactions were performed in StepOnePlus Real-Time

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Gene Name</th>
<th>Primer Forward (F) and Reverse (R)</th>
<th>Exon</th>
<th>Amplicon size (bp)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoe</td>
<td>Apolipoprotein E</td>
<td>F: AGCTGCCAGAGCTCCCAAGTC R: CCGCCGTCACCTCTTTTAC</td>
<td>3</td>
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<tr>
<td>Mapt</td>
<td>Microtubule Associated protein TAU</td>
<td>F: CCAGTAAAGGCCGCTACAGA R: GGACGTTGCTAAGATCCAGTT</td>
<td>9</td>
<td>150</td>
</tr>
<tr>
<td>C3</td>
<td>C3</td>
<td>F: GACCTGCGACTGCTCCCTACTT R: CGGTCTTCAACCACATCTTTTC</td>
<td>20</td>
<td>272</td>
</tr>
<tr>
<td>Ps-1</td>
<td>Presenelin-1</td>
<td>F: CATTCCAGAAGACCCGGAGGA R: TCAGATCAGGAGTGAACCC</td>
<td>5</td>
<td>261</td>
</tr>
<tr>
<td>B-actin</td>
<td>Beta actin</td>
<td>F: TGTCACACAAGGGGGAGA R: GGGTGTTGGAGGTCCTCAA</td>
<td>3</td>
<td>165</td>
</tr>
</tbody>
</table>

*Amplicon size is represented by the number of base pairs (bp).
PCR System (Thermo Fisher Scientific). For each reaction, 50 ng cDNA was used (2 µL), adding 1x RT2 SYBR Green Mastermix (10 µL), 0.2 mM of the forward and reverse primers (except for C3 and Ps-1 reactions, in which 0.1 mM was used) and 7.2 µL nuclease-free water (Thermo Fisher Scientific), totaling 20 µL reaction volume. Amplification was performed by 40 cycles at 94°C for 15 sec and 60°C for 1 min.

Data analysis

The dependent measures analyzed in the EPM included: time spent and absolute number of entries both in the open arms (provided indexes risk behavior and anxiety) and closed arms (locomotor activity), as well as in the center square (indexes of risk assessment and decision making).

All data were analyzed by repeated measures analysis of variance (ANOVA), with groups as the between and sessions and trials as the within-subject’s factors. Separate ANOVA’s were performed for each measure.

Gene expression analyses were performed using the data generated by StepOne Software v.2.3 (Thermo Fisher Scientific). For the statistical analyses, fold change (2^(-ΔΔCt)) of each sample was used as the relevant dependent variable with the administration of STZ (present or not) and time of testing (short or long-term effect) representing the treatment, or “categorical factors”, using ANOVA analysis. When necessary, Tukey’s post hoc test was used to verify specific differences among experimental groups. All the analyses were performed using Statistica 8.0 software (StatSoft, Inc., 2011). P values ≤ 0.05 were considered significant.

RESULTS

Intracerebroventricular STZ increases risk behavior and memory impairments

Animals from both phases were exposed to the EPM, where behavioral parameters such as time percentage and absolute number of entries in the open and closed arms (anxiety, episodic memory), as well as number of crosses in the center square (risk assessment, decision-making) were measured and recorded.

STZ group presented increased time percentage (Fig. 2A) and absolute number of entries (Fig. 2B) into the open arms when compared with the control group (group effect: F(1,27)=11.71; p<0.01), suggesting a risk behavior associated with anxiety. Additionally, the STZ group presented an increased time percentage in the center square (Fig. 2C) in comparison to the control group (group × trial effect: F(1,27)=21.23; p<0.01), indicating a risk assessment-related behavior and episodic memory impairment. Additionally, STZ exposure resulted in an increased number of crosses in the center square in both trials (Fig. 2D) when compared with the control group (group × trial effect: F(1,27)=10.08, p<0.01), emphasizing an increased risk behavior and memory impairment associated with icv STZ administration.

The proportion of entries into the closed arms was significantly higher in STZ group (group effect: F(1,27)=4.29, p=0.04) than the control (Fig. 2E), indicating high locomotor activity.

Although no significant differences were found between phase 1 and phase 2, both STZ groups showed alterations in anxiety, memory and risk assessment related behaviors.

Ps-1, Apoe and Mapt gene expression were not affected by STZ administration

Considered as part of important processing pathways of β amyloid peptide, Ps-1 (p=0.40) (Fig. 3A) and Apoe (p=0.24) (Fig. 3B), genes which are strictly related to the formation of Aβ plaques were not significantly affected by the STZ treatment during the evaluation period.

Besides Aβ plaques, neurofibrillary tangles are also an important pathological characteristic for AD. Mapt encodes TAU protein and its upregulation has been observed in both AD human brains and animal models. Although this study has not found a significant difference between the treatment groups, there was a trend in which the STZ group showed a marginally increased expression of Mapt (group × phase effect; F(1,14)=3.33, p=0.08) in phase 1 (Fig. 3D).

Intracerebroventricular administration of STZ increases C3 expression

Neuroinflammation is one of the most remarkable events that accompanies neurodegeneration processes. C3 expression showed a significant increase in STZ group (Table II) when compared with the control group, specifically in the first phase (group × phase effect: F(1,14)=8.57, p=0.01) (Fig. 3C).

DISCUSSION

This study showed that icv-STZ administration increases risk related behavior and induces memory im-
Streptozotocin increases hippocampal C3 gene expression

Fig. 2. Short-term (phase 1) and long-term (phase 2) behavior effects evaluated by EPM. Behavior effects of icv citrate buffer (white bar) or STZ (gray bar) injections in the EPM test represented by mean values ± SEM of trials. Data were analyzed by repeated measures analysis of variance (ANOVA) with the groups (control and STZ groups) as between-subject factor, and trials (trial 1 and 2) and phases (phases 1 and 2) as within-subject factor. (*) Represents a significant difference (p<0.05). Animals from different phases showed the same behavior pattern with no significant statistical difference between them. The group that received the STZ showed in both an increased time percentage (A) and number of entries (B). Additionally, the STZ group showed an increased time percentage in the center square (C) in comparison to control group. Moreover, animals which received STZ exhibited a significant increase in the number of crosses in center square (D). The proportion of entries into closed arms was also higher in STZ group (E). P value for each parameter is plotted under the graphic.
pairments as well as increases $C3$ gene expression in the hippocampus of Wistar rats.

The EPM is usually used as a tool to evaluate anxiogenic or anxiolytic effects of drugs (Montgomery and Monkman, 1955). However, over the past years, studies have demonstrated other behaviors related to anxiety that can also be analyzed by the EPM (Carobrez and Bertoglio, 2005). The task has been performed to elucidate the understanding of emotional biological basis related to learning and memory, defensive behavior and anxiety disorders, such as phobia and post-traumatic stress (Lamprea et al., 2000; Carobrez et al., 2001; Rasmussen et al., 2001). The EPM can be also used to evaluate episodic memory retention when animals are exposed more than once to the equipment. When the animal is exposed to an open and elevated area (open arms) it is consolidated an aversive memory conditioned to a specific context (Sharma and Kulkarni, 1992; Tanyeri, 2014; Mutlu et al., 2015). Once the

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Phase</th>
<th>Fold Change</th>
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<tbody>
<tr>
<td>Control</td>
<td>1</td>
<td>2.64</td>
</tr>
<tr>
<td>STZ*</td>
<td>1</td>
<td>6.12</td>
</tr>
<tr>
<td>Control</td>
<td>2</td>
<td>2.30</td>
</tr>
<tr>
<td>STZ</td>
<td>3</td>
<td>3.15</td>
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* Represents a significant increase ($p<0.01$) in STZ group of Phase 1.

Fig. 3. Quantitative expression analysis of AD related genes in control (white bar) and STZ groups (gray bar). Quantification data for all genes were normalized using β-actin as reference gene. Data are represented by mean values ± SEM and analyzes were performed by ANOVA. (*) Represents a significant difference ($p<0.05$). The relative Fold changes of mRNA expression are shown in "y axis" in relation to control phase 1 sample (defined as 1) represented by dashed line. $Ps-1$, $Apoe$ and $Mapt$ gene expression did not showed significant difference between both group and phase. However, $C3$ showed a significant increased expression in STZ group, specifically in phase 1 ($p<0.01$).
animal is exposed to the maze during the first trial, the scores for open arms exploration usually decrease when the animal is re-exposed to a second trial, suggesting an episodic memory consolidation.

Regarding the memory impairments, the data obtained in this study corroborate with findings from previous rodent studies that received icv-STZ. For example, an increased time spent in the open arms had also been observed in different studies using animal models for Alzheimer’s disease. Jawhar et al. (2012) described an age-dependent increase in the 5xFAD mice. Oakley et al. (2006) described a double transgenic APP/PS1 mouse model that co-expresses five AD mutations leading to accelerated plaque formation and increased Aβx-42 production, when compared with wild types. Flanigan et al. (2014) reported this behavior in the same animal model and genotype and suggested that it did not reflect reduced anxiety, but rather abnormal avoidance of the closed arms on the part of transgenic and within-session habituation to the closed arms on the part of wild type controls.

In the present study, rats receiving STZ also showed an increased time percentage in the center square of EPM in relation to the control group, suggesting a compromised risk assessment behavior. Rodgers et al. (1997) mentioned that risk assessment is an important behavioral measurement, closely related to fear and anxiety. The biological function of acts and postures related to risk assessment is to advise behavioral strategies in potentially dangerous situations (Blanchard et al., 1991; 1993). Therefore, our results suggest that STZ induced impairments on risk assessment in rats when exposed to dangerous and aversive environmental conditions.

Although previous studies had shown that repeated testing did not modify the baseline measures of open arm exploration (Lister, 1987; File et al., 1990) currently, there are evidences that repeated testing increases the open arms avoidance during the re-test (Bertoglio and Carobrez, 2000; 2002), suggesting that there is a learning and memory process related to the memory consolidation based on the previous aversive experience. In the current study, animals from the control group presented increased avoidance to the open arms (less open arm exploration) in the re-test as described by (Bertoglio and Carobrez, 2000; 2002). However, STZ group showed the same exploration pattern to the open arms in the second trial when compared to the first exposure, indicating a risk related behavior and memory impairment, since the animals did not remember the aversive conditions experienced in the first trial.

Chen et al. (2012) reported that the most prominent brain abnormality in the icv-STZ mouse, when compared to transgenic model (3xTg-AD), was the neuroinflammation process. Current research corroborates this conclusion, since upregulation of C3 mRNA suggested an acute neuroinflammation in the icv STZ model. Chen et al. (2012) also suggest that the neuroinflammation was associated to oxidative stress induced by STZ administration. In fact, several reports have showed that STZ induces oxidative stress in the brain and that oxidative stress is usually associated with inflammation in a process known as neuroinflammation (Kamat et al., 2016). Indeed, Rai et al. (2014) found an increased level of ROS and nitrite in synaptosomal preparation of hippocampus and cortex, indicating oxidative stress in the brain areas involved with the regulation of memory functions. They also observed an increase of mRNA and protein expression in markers of glial activation and elevated levels of pro-inflammatory cytokine (TNF-α) in brain areas of icv STZ.

There are different patterns of gene expression during AD progression since it is a multifactorial disease (Miller et al., 2013). Changes in gene expression of distinct brain regions affected by the disease are common and usually unpredictable. For this reason, approaches that allow investigation in a molecular level (such as qRT-PCR) are essential to understand molecular pathways involved in AD, which can provide new insights about potential therapeutic targets with molecular characteristics (Ding et al., 2014). Therefore, it is important the elucidation of gene expression in a mRNA level, allowing to explore the dynamics between genes and proteins.

CONCLUSION

Therefore, the present study showed that icv STZ animal model presented impairments in behavioral functions such as anxiety, episodic memory and risk assessment. One month after drug administration, C3 gene, which has an important role in inflammation, showed an increased expression in the STZ group, indicating the presence of a key inflammatory process related to the degenerative events on hippocampal formation. Moreover, it is important to highlight that plaque load may not be the best early diagnostic marker of AD. Therefore, other markers, such as the neuroinflammatories, should be explored to enable the track of AD progression.

ACKNOWLEDGMENTS

Authors would like to thank Universidade Positivo for the institutional and financial support.
REFERENCES


Streptozotocin increases hippocampal C3 gene expression


Supplementary Fig. 1. (A): RNA was extracted from rat hippocampus following PureLink RNA Mini Kit® (Thermo Fisher Scientific) with trizol reagent, as recommended by manufacture, and its presence and integrity was evaluated by agarose gel electrophoresis 1.5%. Representative sample of each experimental group was tested: 1: Control group phase 1, 2: STZ group phase 1; 3: Control group phase 2; 4: STZ group phase 2. RNA samples presented a satisfactory profile, considering the presence of rRNA bands (28S and 18S). (B): Agarose gel electrophoresis 1.5% was performed in order confirm the fragment size generated by each primer pair. 1: 1kb DNA ladder Plus (Invitrogen); 2: PCR product amplified with β-actin (165 bp); 3: Apoe (150 bp); 4: C3 (272 bp); 5: Mapt (150 bp); 6: Ps-1 (161 bp). All samples showed an expected fragment size accordantly to each primer used.
Supplementary Fig. 2: Melting curve of Apoe, Ps-1, C3 and Mapt genes. The amplification specificity of each qRT-PCR assay was confirmed by melting curve analysis. Each primer pair showed a single peak, indicating that the primers amplified only a specific PCR product.