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MOLECULAR ANALYSIS OF GENE EXPRESSION IN WISTAR RATS SUBMITTED TO INTRACEREBROVENTRICULAR STREPTOZOTOCIN (STZ-ICV) AND EXPERIMENTAL TREATMENT WITH DIMETHYL SULFOXIDE (DMSO)

Valter Malaguido Climaco
Universidade Positivo
Curitiba - Paraná - Brazil

Rebecca Marty Pimentel Machado
Universidade Positivo
Curitiba - Paraná - Brazil

Márcia Regina Pincerati
Universidade Positivo
Curitiba - Paraná - Brazil

Estêvão Cabral Santos
Faculdade Evangélica Mackenzie do Paraná
Curitiba - Paraná - Brazil

Ilton Santos da Silva
Universidade Positivo
Curitiba - Paraná - Brazil
Faculdade Evangélica Mackenzie do Paraná
Curitiba - Paraná - Brazil

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Abstract: Alzheimer disease represents the most frequent neurodegenerative and prevalent process in the adult population related to genetic and environmental factors, with no curative treatment even with current technological innovations. Thus, it is important to search for therapeutic proposals that minimize or even interrupt the neurodegenerative processes common of the disease. Dimethyl sulfoxide (DMSO) has high molecular versatility, with a potential neuroprotective effect. Neurodegeneration induced by intracerebroventricular streptozotocin (STZ-icv) in rodents leads to a neuropathological phenotype similar to sporadic Alzheimer disease. The aim of this study was to assess the potential neuroprotective effect of DMSO on memory impairments induced by STZ in Wistar rats, with molecular analyses performed by hippocampal extraction through qPCR and assessing the expression profile of genes related to neuroinflammation (interleukin 1β; IL 1-β, tumor necrosis factor-α: TNF-α), reactive species of mitochondrial oxidative stress; catalase (CAT), and an activator of adulthood neurogenesis (brain-derived neurotrophic factor; BDNF), where a trend in reduction of neuroinflammation was observed through lower expression of IL1-β, TNFα, mitochondrial oxidative stress with degradation of CAT in the scavenging of reactive oxygen species, and an increase in the expression of BDNF at the dose of 1.5 g/kg DMSO in the treatment group, related to a cellular environment favourable to neuroprotection and cell survival upon induction of neurodegeneration.
Keywords: Alzheimer disease. Dimethyl sulfoxide. Streptozotocin.

INTRODUCTION

Alzheimer's disease is the most frequent and prevalent neurodegenerative process common to aging, affecting 6% of the population over 65 years and has no curative treatment [1]. Clinically, explicit memory functions dependent on hippocampal formation are compromised, besides other cognitive alterations [2].

Alterations extend to the medial, prefrontal and parietal temporal cortex with the deposition of cytoplasmic neurotoxic amyloid protein in beta-conformation (Aβ 42), and neuronal. Impaired glucose metabolism and insulin signalling occur (activating the enzyme GSK-3β), as well as increased production of pro-inflammatory cytokines, loss of the blood-brain barrier and mitochondrial oxidative stress [6]. Irregular protein conformation promotes their insolubility and aggregation, with consequent loss of signalling of the autophagic system, protein folding proteins, chaperones [7]and expression of neurotrophins [8].

Intracerebroventricular(icv)streptozotocin (STZ) induces a neuronal state equivalent to Alzheimer disease. Its mechanism of action involves an insulin metabolic blockade and glucose transport via GLUT123 transporters. In addition, it biochemically facilitates amyloid formation (Aβ 42) and hyperphosphorylation of the TAU protein [9] which contributes to the reproduction of the pathological characteristics of the disease. Experimentally, it is possible to observe loss of memory in animals that received STZ-icv in Morris water maze tasks, because the integrity of the hippocampal formation is needed to fulfil tasks of spatial memory [10].

Molecular gene expression analysis have shown that pathological activating cycles of neuroinflammation (IL-1β, TNF-α), mitochondrial oxidative stress (Catalase-CAT), brain-derived neurotrophic factor (BDNF) [5,11] are reverberant and self-sustained processes of the disease. DMSO therapy has demonstrated versatility in the

molecular interaction with water [12] and interconversion of the water-DMSO complex [7]. It acts in the superficial hydration of proteins, modifying H+ connections, and facilitates the action of protein chaperones. In addition, it reduces inflammatory activation and promotes the scavenging of reactive species from the mitochondrial cycle possessing antioxidant action [13,14].

Rodent studies by Jacob and de la Torre [15] have demonstrated the efficacy of venous DMSO at 40% in spinal cord injury and acute carotid vascular occlusion, while Penazzi et al.[16] in a study with chronic and oral 1% DMSO observed positively modulating budding of pyramidal hippocampal dendritic spines. Another study by Kumar [17] described the competitive inhibition of the cholinesterase enzyme in vitro with satisfactory results.

Thus, the objective of the present study was to assess the potential neuroprotective effect of DMSO on memory impairment induced by STZ in Wistar rats.

MATERIAL AND METHODS

ANIMALS

A total of 42 male 90 days-old Wistar were used in the study. The animals had access to food and water *ad libitum*. The environmental conditions included a 12 hours light/dark cycle and a temperature of 21°C.

STEREOTACTIC SURGERY FOR ADMINISTRATION OF STZ AND TREATMENT WITH DMSO

STZ-icv (3 mg / kg) is administered through stereotaxic surgery in coordinates according to Paxinos and Watson [18]. For group 1 (control), buffer solution is injected into the same volume used for the administration of STZ.

After the administration of STZ (24h), animals in groups 3 and 4 receive doses of DMSO intraperitoneally during 5 consecutive days. The control group received the same doses and volumes of saline solution at 0.9% via i.p. Thus, four distinct groups were formed:

- Group (1) saline control: Animals injected with citrate buffer solution (the same used to dilute the STZ) via icv and 0.9% saline solution i.p;
- Group (2) STZ: animals that received STZ via icv and 0.9% saline i.p;
- Group (3) STZ + DMSO 0.75g/Kg: group that received STZ via icv and DMSO via i.p. at a concentration of 0.75g/kg at 40%, diluted in saline 0.9%;
- Group (4) STZ + DMSO 1.5 g/Kg: group that received STZ via icv and DMSO via i.p. at a concentration of 1.5 g/Kg at 40%, diluted in 0.9% saline.

The choice of doses and procedures were based on the studies of Bardutzky J et al (2005) and Jacob SW, dela Torre JC (2009).

RNA EXTRACTION AND GENE EXPRESSION OF INTERLEUKIN 1-B (IL-1B), CATALASE (CAT), TUMOR NECROSIS FACTOR-ALPHA (TNFA) AND BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF)

The animals were euthanized by constant inhalation of isoflurane and the brains dissected for the bilateral withdrawal of the hippocampus. Soon after, the samples were stored in preservative solution (RNA later - Thermo Scientific) at a temperature of -20º C. The SV total RNA Isolation Kit (Promega) was used to extract RNA, according to the manufacturer's instructions. After extraction, the RNA was quantified and its purity assessed by spectrophotometry.

One microgram of total RNA was used for cDNA synthesis, using the Kit High Capacity cDNA Reverse Transcription Kit (Applied biosystems). The cDNA was then submitted to the quantitative polymerase chain reaction

(RT-qPCR) to evaluate the expression profile of the CAT, IL-1β, TNFα and BDNF genes in the hippocampus of the different groups.

The Power Up SYBR Green qPCR master mix (Thermofisher) was used according to the manufacturer's instructions in a Step One Plus thermocycler (Life Technologies) to determine the levels of expression of the mRNA in different groups.

DATA ANALYSIS

The data obtained were submitted to normality tests and Repeated Measurements Analysis of Variance (ANOVA). Molecular parameters were considered as dependent variables and the experimental groups as independent variables with significance level set at 5% and the Tukey's post hoc test was used for multiple comparisons, to identify the cause of the differences.

RESULTS

EVALUATION OF GENE EXPRESSION BY REAL-TIME PCR

Specific gene signatures establish a self-sustained system in neurodegeneration. Pro-inflammatory activation and overexpressed oxidative stress markers, transcribed from underexpressed neurotrophic markers mark the disease at the molecular level. Neutralizing process-defining steps is critical in curative therapy.

ANALYSIS OF IL-1B EXPRESSION

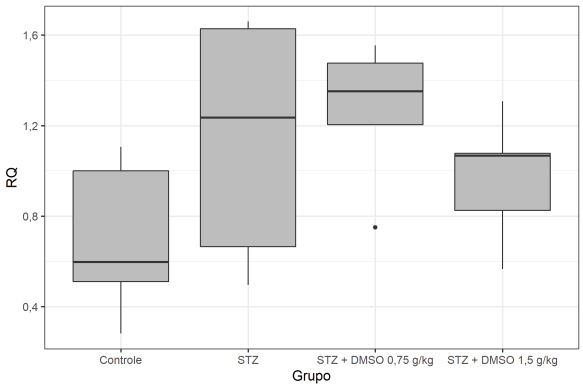

Figure 1 - Boxplots of the variable of interest IL1-β, per group. – Note the trend of a reduction in the expression of IL1-β in the G4 group compared to the STZ groups (G2, G3, and G4). Grupo=Group; RQ=Relative Quantification; Controle=Control

The representation of the means of the variables of interest for each group in relation to IL1-β indicate that the control group and STZ + DMSO 1.5 g/kg displayed in general, lower expression compared to other groups evaluated, while the groups STZ and STZ DMSO + 0.75 g/kg presented the highest values, the latter with less dispersion.

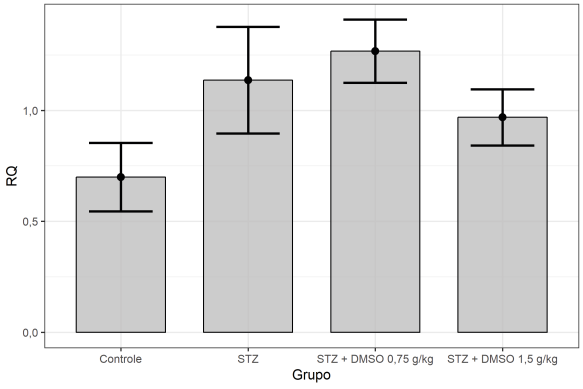

Figure 2 - Bar graph of the mean (± standard error) expression of IL1-β by group – Note the trend of a reduction in expression of IL1-β in G4. Grupo=Group; RQ=Relative Quantification; Controle=Control

The main effect of the groups was not significant ($F_{3,16}$ = 2.039, p = 0.149), at 5%

significance, indicating that the means did not differ significantly between the groups.

ANALYSIS OF TUMOR NECROSIS FACTOR-ALPHA (TNFA) EXPRESSION

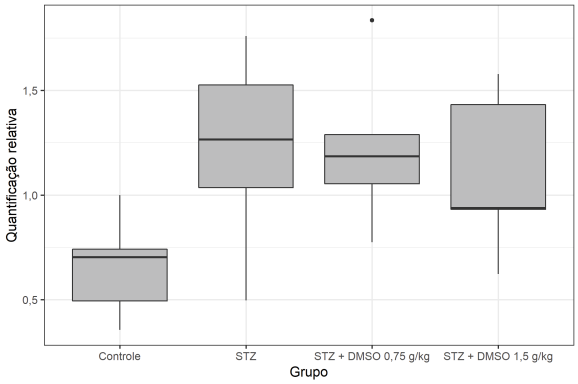

Figure 3 - Boxplots of the relative expression of TNFα per group. - Note the trend of a reduction in expression of TNFα in G4 compared to groups STZ (G2, G3, G4). Grupo=Group; RQ=Relative Quantification; Controle=Control

The gene expression results of TNF indicate that the control group had lower expression compared to other groups evaluated, while the groups STZ and STZ DMSO + 0.75 g/kg presented the highest values, the latter with less dispersion.

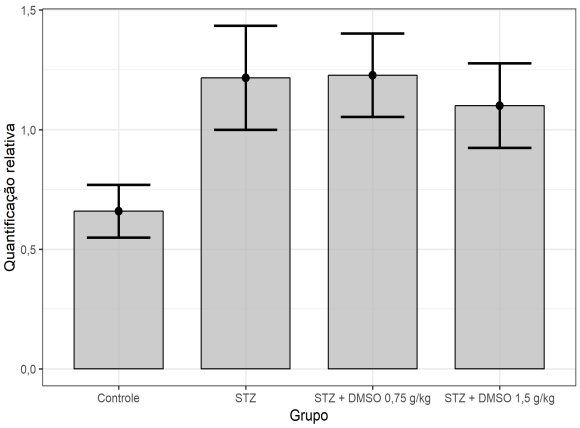

Figure 4 - Bar graph of the mean (± standard error) expression per group of TNFα.- Note the trend of a reduction in expression of TNFα in the STZ groups in G4.

The comparison of the relative quantification (response variables) between the groups (independent variable) show that the main effect of the groups was not significant ($F_{3,16} = 2.366$, $p = 0.109$), at 5% significance, indicating that the means did not differ significantly between the experimental groups.

ANALYSIS OF CATALASE (CAT) EXPRESSION

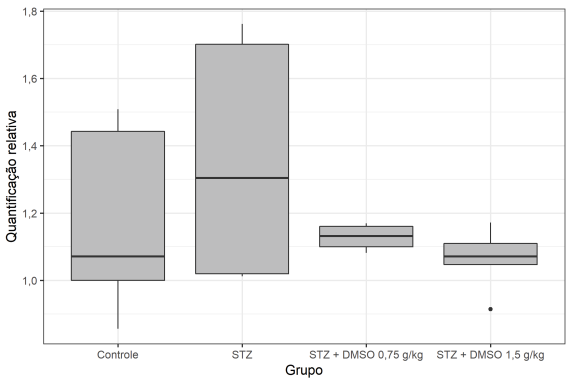

Figure 5 - Boxplot of the relative expression of CAT per group - Note the trend of a reduction in expression of CAT in the groups treated with DMSO (G3 and G4). Grupo=Group; Quantificação Relativa=Relative Quantification; Controle=Control.

The results of the relative quantification of CAT indicates that the group STZ + DMSO 1.5 g/kg have, in general, lower expression compared to the other groups, followed by group STZ DMSO + 0.75 g/kg. On the other hand, groups STZ and control presented the highest values, being more dispersed.

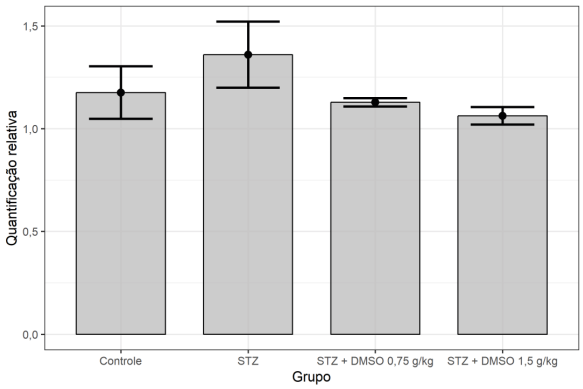

Figure 6 - Bar graph of the mean ((± standard error) expression per group of CAT.- Note the trend of a reduction in expression of CAT in the groups treated with DMSO (G3 and G4).). Grupo=Group; Quantificação Relativa=Relative Quantification; Controle=Control.

The main effect of the groups was not significant ($F_{3, 15}$ = 1.361, p = 0.293), at 5% significance, indicating that the means of the relative quantification did not differ significantly between the experimental groups.

ANALYSIS OF BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF) EXPRESSION

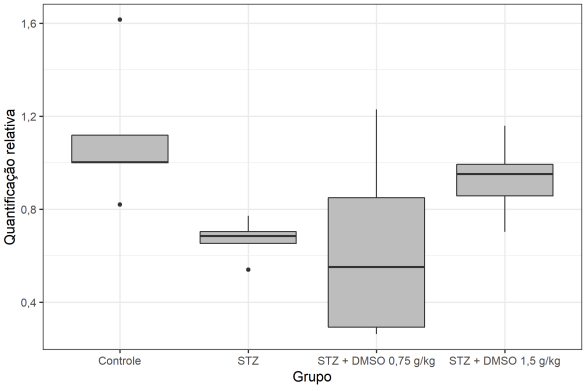

Figure 7 – Boxplot of the relative expression of BDNF per group. – Note the trend of an increase in the expression of BDNF in the treatment group with DMSO (G4).). Grupo=Group; Quantificação Relativa=Relative Quantification; Controle=Control.

The relative quantification of BDNF per group indicate that the control group had, in general, higher values compared to other groups evaluated, while the groups STZ and STZ DMSO + 0.75 g/kg have the lowest values, with the latter presenting higher dispersion and the main effect of the groups was significant ($F_{3,16}$ = 3.477, p = 0.041), indicating that the means differ significantly in the group STZ DMSO + 0.75 g/kg (0.64), alone, with no differences in the other experimental groups.

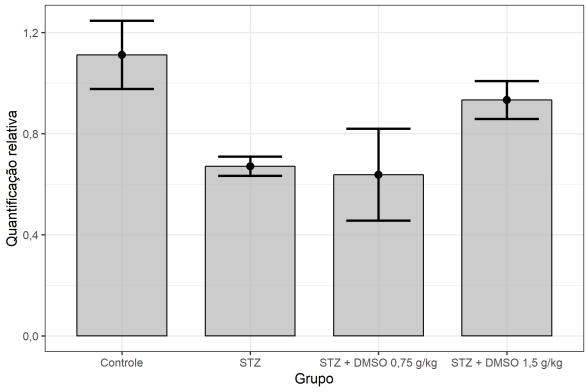

Figure 8 - Bar graph of the mean (and standard error) expression of BDNF per group. - Note the trend of an increase in the expression of BDNF in the treatment group with DMSO (G4).). Grupo=Group; Quantificação Relativa=Relative Quantification; Controle=Control.

Note that the experimental group DMSO 1.5 g/kg displays a similar gene expression of BDNF of the control group, thus, being of interest in the proposed neuroprotection therapy.

DISCUSSION

Knezovic et al. [19] studied structural and neuropathological temporal markers of the STZ-icv model in 9-month old rodents. The results showed a predominant cognitive impairment in the memory domain, characterized by loss of CA1-CA3 hippocampal neurons and neurofibrillary changes, thinning of the parietal cortex and corpus callosum, more evident at 3 months

of exposure, with progressive extension in the hippocampus.

Similarly, in patients with prodromal Alzheimer's dementia, compromised connectivity networks modify the processing of specific functions such as attention and episodic memory. Late motor impairment and spatial memory are also observed according to Koch et al. [20].

Neuroinflammation, as an amplified process in Alzheimer's disease, was characterized by the gene expression of IL1-β and TNFα as pro-inflammatory cytokines.

De Lucca et al. [21] associate the role of neurons, glial cells (astrocytes and microglia) and extracellular matrix in the homeostasis of the neurovascular unit, implying that the rupture of the blood-brain barrier projects neuropathological progression [22,23]. Nevertheless, this is limited to the production of anti-Aβ antibodies in the elimination of amyloid oligomers [24] and is dependent on chronic neuroinflammation, according to Shaftel et al. [25], associated with the recent role of aquaporin 4 channels in compromised glial-lymphatic traffic [14,26]

Amyloid deposits and the hyperphosphorylated state of Tau protein are neuroinflammation factors in neurodegeneration [1]with astrocytic and microglial activation, and promotion of secretion of pro-inflammatory cytokines type IL-1β, TNFα, IL-6, Infγ, membrane metalloproteinases, and nuclear transcription factor NFK-β, contributing to the recruitment of systemic lymphocytes via type II major histocompatibility complex (MHC II) as well as the abnormal processing of micro-RNAs (mir155; mir204) [1,27].

The inflammatory activation affects immunological constituents with the blockade of glycolytic enzymes, mitochondrial impairment, cell survival signalling pathways and loss of axonal synaptic density [27-29].

The analysis of the expression of inflammatory genes IL1-β and TNFα in the present work did not reveal statistical differences between experimental groups, but a reduction in gene expression of IL1-β and TNFα was observed in the group treated with DMSO at the dose of 1.5 g/kg, which can relate to a cytoprotective role in the STZ-induced inflammatory milieu.

Studies of Mao et al. [30] demonstrate that the abnormal accumulation of amyloid oligomers was reduced by the activation of mitochondrial antioxidant enzymes (catalase).

The STZ-icv model promotes significant elevations of the specific activity of catalase (CAT) expressed in hippocampal tissue, according to Sofic [31]. The extensive generation of hydrogen peroxide (H_2O_2) is neutralized by CAT, capable of degrading up to 42,000 molecules per second.

Neurotoxic changes converge to the permeabilization of mitochondrial membranes, associated with intracellular calcium influx, with involvement of oxidative phosphorylation in the respiratory chain and overexpression of reactive oxygen and nitrogen species [32].

These elements favour the flow of electrons from NADH to molecular oxygen in the production of ATP, reduce antioxidant and neuroprotective elements such as glutathione peroxidase (GSH-Px), catalase (CAT), and superoxide dismutase (SOD) [33] and the cell energy reserves with insufficient signal transduction pathways in plasticity, polarization and immunomodulation.

Our results showed no significant variation in the gene expression of catalase in the experimental groups, observing trends of neutralization of the enzymatic activity in the capture of reactive oxygen species in the groups treated with DMSO (G3 and G4) through a lower gene expression of CAT.

In Alzheimer disease, a reduction in the

levels of BDNF also occurs, with dysregulation of transcripts and protein expression, affecting synaptic plasticity, axonal-dendritic budding and neuronal survival [34].

Brain-derived neurotrophic factor (BDNF) is an important member of the neurotrophin family (growth factors) activated via specific receptors, tropomyosin kinase (TrkB) and p75.

The reduction in BDNF signalling compromises spatial memory, hippocampal synaptic plasticity and maturation, neurogenesis, and long-term potentiation (LTP) in the formation of dendritic spines [35], essential factors for the consolidation of explicit memory.

Amyloid oligomer aggregates in Alzheimer disease negatively modulate p75 and TrkB receptors, which reduces the PI3K-GSK3β -AKT neuronal survival signalling pathways, cause the rupture of microtubules with hyperphosphorylation of Tau protein (Thr 231) and compromise protein expression in synaptic plasticity [36-38].

The expression of BDNF in hippocampus of chemically induced rats improves cognitive impairment in learning and memory, involving the PI3K-AKT-GSK3β pathway, highlighting its potential protective effect on neuroregeneration in Alzheimer's disease [36]. Its positive regulation improves synaptic and cholinergic plasticity, facilitating the maturation of neuronal budding, especially in the early stages of neurodegeneration [34]. The present study demonstrated an increase in the expression of BDNF at doses of 1.5 g/kg (G4) relating to a potential neuroprotective role.

The expression of genes involved with neuroinflammation, IL1-β and TNFα, mitochondrial oxidative stress with CAT, and neurotrophic activation with BDNF show that there was no statistical effect in pairing of groups. However, a trend towards a reduction in neuroinflammation and neutralization of oxidative stress were observed, which promote a more favourable cellular environment at the molecular level, corroborating the increase in the comparative expression of BDNF in animals treated with DMSO 1.5 g/kg, with partial effects on proinflammatory modulation, reduction of mitochondrial oxidative stress and neurotrophic activation.

Biotechnology has contributed to the search for biologically active molecules, for example in new applications of central cholinesterase inhibitors [39], β-amyloid peptide and Tau protein immunotherapy [40], neuroinflammation [41], nanotechnological applications [42], neurotrophic factors [43] and modulation of receptors for advanced glycation [44]. However, the effects on the decrease of neurodegenerative progression are still little studied.

The present study aimed at evaluating the effect of DMSO as a new molecular basis of therapy in the central nervous system, since its chemical and molecular properties may rescue cellular functions restricted by the neurodegenerative process.

Singh [45] defined the term pharmacological-hybrid for shared molecular action functions in therapeutic targets in Alzheimer's disease, corroborating Weinreb et al. [46], who observed the neuroprotective effects of hybrid agents on more than one potential neurodegenerative therapeutic target. Farkas [47] proposed that DMSO has neuroprotective and modulating characteristics of the central nervous system, and Denko [48], in studies of the distribution of DMSO in experimental animals, showed an equivalent distribution in the central nervous system without cumulative effects and elimination in 12 to 36 hours.

Actions in spatial memory, improved hippocampal synaptic density and plasticity, mainly in the CA1 stratum pyramidal cells

in studies by Penazzi [16], are indicative of the pharmacological relevance of DMSO, besides the inhibition of the enzyme GSK-3β and reduction of amyloid deposits by Jiang [49]. Antioxidant properties in studies of Sanmartin-Suarez [50] demonstrate the versatility of DMSO applied to molecular neurodegeneration.

Amid the conformational neurodegenerative proteinopathies, one study by Shaked et al. [51] can be highlighted, showing positive effects of DMSO in hamsters infected with the scrapie prion protein (Prp sc) and relieving the symptoms of the disease in infected animals [51], reinforcing Pruisner studies [52] on the modulation of protein folding by DMSO.

The present study demonstrated trends in pro-inflammatory modulation and mitochondrial oxidative stress, modifying sequential neurodegenerative reverberation activation and promoting neurotrophic rescue tendencies with BDNF by treatment with DMSO at the dose of 1.5 g/kg.

CONCLUSIONS

Neurodegeneration represents a complex of activating determinants in stages of a sustained process towards irreversible cell death.

The study of the therapeutic proposal in the use of DMSO in the STZ model did not reveal a significant improvement in behavioral and spatial memory tests.

The neuroinflammatory and oxidative mitochondrial gene expression revealed tendencies of neuroprotection with treatment with DMSO at 1.5 g/kg, where a molecular physiologically favourable environment was created, corroborating with the expression of BDNF by this same group, being a significant element in hippocampal synaptic plasticity and neuronal survival.

Further studies are needed in the current neuroprotective proposal, especially with different doses, time and administration, in the extensive use of the product, thus being able to intensify more robust and effective therapeutic responses.

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