Research Report

Effects of a Nutritional Formulation Containing Caprylic and Capric Acid, Phosphatidylserine, and Docosahexaenoic Acid in Streptozotocin-Lesioned Rats

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Abstract

Background: It has been studied that nutrition can influence Alzheimer’s disease (AD) onset and progression. Some studies on rodents using intraventricular streptozotocin (STZ) injection showed that this toxin changes cerebral glucose metabolism and insulin signaling pathways.

Objective: The aim of the present study was to evaluate whether a nutritional formulation could reduce cognitive impairment in STZ-induced animals.

Methods: The rats were randomly divided into two groups: sham and STZ. The STZ group received a single bilateral STZ-ICV injection (1 mg/kg). The sham group received a bilateral ICV injection of 0.9% saline solution. The animals were treated with AZ1 formulation (Instanth® NEO, Prodiet Medical Nutrition) (1 g/kg, PO) or its vehicle (saline solution) for 30 days, once a day starting one day after the stereotaxic surgery (n = 6–10). The rats were evaluated using the open field test to evaluate locomotor activity at day 27 after surgery. Cognitive performance was evaluated at day 28 using the object recognition test and the spatial version of the Y-maze test. At day 30, the rats were anesthetized with chloral hydrate (400 mg/kg, i.p) and euthanized in order to evaluate IBA1 in the hippocampus. The differences were analyzed using one-way ANOVA with Bonferroni’s or Kruskal Wallis with Dunn’s post-hoc test.

Results/Conclusion: STZ-lesioned rats present memory impairment besides the increased microglial activation. The treatment with AZ1 formulation reversed the memory impairment observed in the object recognition test and Y-maze and also reduced IBA1 in CA1 and DG.

Keywords: Alzheimer’s disease, IBA1, Instanth® NEO, object recognition test, streptozotocin, Y maze

INTRODUCTION

Alzheimer’s disease (AD) is a neurodegenerative disease and the most common form of dementia. It is mainly characterized by progressive and slow memory loss, especially sporadic memory (related to recent events) and spatial disorientation. AD presents common histopathological findings, evidenced by protein accumulations. These findings are described as intracellular neurofibrillary tangles in cortex and hippocampal neurons caused by tau protein hyper-phosphorylation, and as amyloid-β extracellular...
filamentous aggregation, also called senile plaques [1–7]. Disease progression also causes hippocampal, neocortex, and amygdala atrophy, mainly seen in postmortem examinations. Such protein dysfunctions also result in a cascade of events involving neuronal loss, neuroinflammation, glial reactivity, cognitive and functional disorders [1–6], and decreased cerebral glucose metabolic rate, which worsens the cognitive status [8–13].

Many of the signals seen in AD, such as severe cognitive impairment, neuroinflammation, and oxidative stress, are also described after intracerebroventricular (ICV) injection of streptozotocin (STZ), being validated by several studies [14–18]. This toxin, produced by the bacterial strain Streptomyces achromogenes, is commonly used for animal models of type II diabetes for its ability to act on β-pancreatic cells and cause insulin resistance. However, STZ can cause cerebral glucose metabolic rate dysfunction by inhibiting insulin receptors when administered ICV at sub-diabetogenic doses. These factors trigger neuroinflammation, glial activation, and neuronal death, as well as a cognitive decline related to loss of spatial and recognition memory, very similarly to AD symptoms [15–18].

AD also presents abnormal complications of neuronal membrane lipids with increased cholesterol to the detriment of phosphatidylserine [19–21]. In this context, oral phosphatidylserine supplementation can cross the blood-brain barrier and increase the brain supply of this nutrient [21]. Some experimental studies showed that phosphatidylserine helped improve cognitive problems and performance on tasks testing short-term learning and memory skills [21].

Another important nutrient in the composition of the neuronal membrane is docosahexaenoic acid (DHA), which has a complementary function to phosphatidylserine supplementation. About 20–30% of phosphatidylserine in gray matter is combined with DHA [22, 23]. Decreased phosphatidylserine DHA in cerebral cortex is associated with AD mild cognitive impairment progression [21, 24]. In addition, the brain is vulnerable to lipid peroxidation, decreasing membrane fluidity, damaging membrane proteins, and resulting in membrane breakdown [25]. In the STZ-induced AD model, Pardeshi et al. [26] demonstrated that DHA improved the efficacy of the pharmacological agent used in the study and increased survival and neuronal memory, decreasing oxidative stress and inflammation.

Diets that induce ketone body formation have been related to preserved cognition and reduced tau protein and amyloid-β pathology in AD animal models [27, 28]. One of the mechanisms responsible for the formation of ketone bodies is the use of caprylic and capric fatty acids [29–31], even when added to regular meals, as they are rapidly absorbed by the portal system and beta-oxidized in the liver, generating excess acetyl-coA, which forms ketone bodies [31, 32]. Ketone bodies cross the blood-brain barrier [33], enter the neurons, and produce ATP in the mitochondria by oxidative phosphorylation [31]. Thus, caprylic and capric acids are also important nutrients in AD.

Although many nutrients seem to be important for brain health in AD, there are no studies evaluating these nutrients combined. The objective of the present study was to evaluate whether a formulation with a unique combination of capric acid, caprylic acid, phosphatidylserine, DHA, vitamins, and minerals can reduce cognitive impairment in STZ-induced animals as well as the neuroinflammatory markers of the model.

**MATERIALS AND METHODS**

**Animals**

This study included 32 young adult (approximately 3 months old) Wistar male rats (Rattus norvegicus) from the Central Laboratory Animal Facility of the Federal University of Paraná (UFPR) weighing 290–320 g at the beginning of the experiment.

Each five animals were kept in a polypropylene box with ad libitum water and food intake in a room with controlled humidity and temperature (22 ± 2°C) and 12 h light-dark cycle (7 a.m.–7 p.m.). The procedures were approved by the Animal Ethics Committee (CEUA) of the UFPR under protocol number 1226.

**Stereotaxic surgery**

The animals were deeply anesthetized with sodium thiopental (30 mg/kg, i.p.) and chloral hydrate (150 mg/kg, i.p.) followed by atropine sulfate (0.4 mg/kg IP) as an anesthetic adjunct to reduce mucus production. The rats were placed in the stereotaxic equipment (David Kopf, 957L model) and positioned according to the following coordinates: anteroposterior (AP): −0.8 mm from the bregma; mediolateral (ML): ±1.5 mm from the midline; and dorsoventral (DV): −3.8 mm from the skullcap [34]. The head region was shaved and a small skin incision
Table 1
Composition per 100 g of the nutritional formulation AZ1

| Composition            | 100 g |
|------------------------|-------|
| Caprylic acid          | 36 g  |
| Capric acid            | 27 g  |
| DHA                    | 327 mg |
| Phosphatidylserine     | 546 mg |
| Minerals               |       |
| Magnesium              | 233 mg |
| Zinc                   | 5.6 mg |
| Selenium               | 102 mcg|
| Vitamins               |       |
| Vitamin D              | 75 mcg |
| Vitamin E              | 56 mg α TE |
| Vitamin C              | 436 mg |
| Vitamin B6             | 36 mg  |
| Folic Acid             | 526 mcg|
| Vitamin B12            | 12 mcg |
| Niacin                 | 31 mg NE |
| Choline                | 835 mg |

mas made to expose the skull. Subsequently, two incisions were drilled in the skull with a dental surgical drill, followed by the insertion of a 30-gauge needle connected to a polyethylene tube adapted to a 10 μL micro syringe (Hamilton, USA) and connected to an infusion pump (Harvard Apparatus, USA).

The lesioned group received bilateral ICV injections of STZ (1 mg/kg total dose) dissolved in sterile saline solution (2 μL per injection site) into the lateral ventricles. The sham group animals underwent the same surgical procedure; however, they received only the diluent (0.9% sterile saline solution) instead of the STZ solution. Animals in STZ groups received a bilateral 2 μL microinfusion of STZ solution (1 mg/kg) dissolved in sterile saline solution. Animals in sham groups underwent the same surgical procedure; however, they received the same amount of 0.9% sterile saline solution instead of the STZ solution. The groups were treated with AZ1 formulation (1 g/kg) or saline solution for 30 days, once a day in the afternoon, starting one day after surgery.

At day 27 after surgery the animals underwent the open field test (OFT) to evaluate spontaneous locomotion and rearing frequency. At day 28 the animals underwent the object recognition (ORT) and the Y-maze tests, which evaluated spatial memory and short-term recognition. Finally, at day 30 the animals were deeply anesthetized with hydrated chloral (400 mg/kg, IP) and euthanized with an intracardiac perfusion process. Subsequently, the brain structures were prepared and immunohistochemically evaluated with the expression of the microglia-specific marker for ionized calcium-binding adaptor molecule (IBA1).

Open field test

The OFT is a behavioral test widely used to measure locomotor and behavioral characteristics in rodents. It is an easy-to-perform test based on physiological concepts well discussed in the literature that is also used to evaluate anxiety and exploratory behavior [35–37].

The test takes place in a white round arena (97 × 42 cm) divided by three concentric circles into three parts subdivided by straight segments into 19 quadrants with a central circle where the animal was carefully placed, allowing it to freely explore the entire area for 5 min. This study analyzed two motor parameters: locomotion (ambulation) and rearing frequency. Locomotion is one of the most analyzed factors [36] and considers the quadrants the animal crosses with all four paws as a measure of motor activity [35]. Rearing is the frequency with which the animal stands on its hind legs and measures exploratory activity [35]. The arena was cleaned prior to each animal test with 20% water-ethanol solution to eliminate odors and substances left by the previous rat.
Object recognition test

The ORT is associated to the ability the animal has to distinguish between known and new objects. Rodents have an intrinsic tendency to spend more time exploring new than familiar objects [38]. This decreased novelty may be related to repeated exposure to a new stimulus that eventually becomes familiar; however, it should not be confused with decreased exploratory interest [38].

The ORT takes place in a square black (to avoid clues) box (100 × 100 × 40 cm) made of wood and positioned in a moderate light environment (20 lux). A camera was positioned above the box for subsequent image analysis in order to avoid interference due to the presence of the handler in the test environment. The test started with an adaptation session in which the animals were placed in the box for 5 min for free exploration of the environment. Another 5 min session was held 24 h later and the test started 1 h after [39]. Session one was a training session. The animals were placed in the test box with two identical objects and had 5 min to explore them before being placed back in the habitat box. One hour after the initial exposure, the animals were placed back in the test box with a new object for 3 min. This session was recorded for later evaluation of how much time the animal spent interacting with each object [40].

The objects used in the test were made of plastic or ceramic and cleaned with 20% ethanol (standardized by the laboratory) after each session to avoid the influence of the smell of other animals [40]. The objects were randomly arranged to reduce the bias of preference for specific place or object. Weights have been added to objects so that the rat could not move or knock them down [17, 41, 43]. All animals were placed in the box facing the same wall in both sessions [39]. A chronometer was used to measure the time the animal explored the objects, which was considered only when the animal smelled an object at a distance less than 2 cm or touched the object with the muzzle. Sitting, rearing, or walking around objects were not considered exploratory behaviors [42].

Exploratory behavior was measured by counting how much time the animal explored each object during the test session [40]. The time spent by the animal exploring the familiar object and the new object will be represented by ‘a’ and ‘b’, respectively. The variable ‘e’ is the sum of the total exploration time of the new and familiar objects during the test session. The variable ‘d’ is an index of discrimination between the new and the familiar objects and was considered a relative measure to correct the exploratory activity of the animal. Thus, the formula was: 
\[ e = a + b; \text{ and } d = (b - a)/e \] [39].

Spatial version of the Y-maze test

In this test the animal was placed in a Y-shaped maze with three identical arms at an angle of 120°. These arms measured 50 × 12 × 27 cm. The maze was placed in a controlled light room (20 lux). A camera was positioned above the maze for subsequent image analysis in order to avoid interference due to the presence of the handler in the test environment.

The test consisted of two steps with a 1 h interval between them: a training and a test session. In the training session, the animals were randomly placed in one arm of the maze but a removable wood door previously positioned restricted the access to one of the other arms. At this stage the animals could freely explore two arms of the maze for 5 min and after were placed back in the habitat box. The test session took place 1 h after. The animals were placed in the same arm where they started the training session but the barrier that prevented the animal from accessing one arm was previously removed, allowing free exploration of all arms for 3 min. This session was recorded and the parameter evaluated was the time the animal stayed in the unknown arm. The maze was cleaned with 20% ethanol between sessions to avoid the influence of the smell of other animals on the test [17, 41, 43].

The time each animal stayed in the unknown arm was corrected by latency to leave the initial arm and time spent in the center of the maze. Animal entrance in one of the arms was considered when it had all paws inside the arm [17, 41, 43].

IBA1 immunohistochemistry

Brain sample processing and immunohistochemistry reactions were developed as described by Bassani et al. [17]. The animals were deeply anesthetized and then euthanized with an intracardiac perfusion of saline phosphate buffered solution (pH 7.4; 1,000 mL/kg; 4°C), followed by 4% paraformaldehyde in phosphate buffered solution (pH 7.4; 1,000 mL/kg, 4°C). Then the brains were removed and placed in 30% sucrose solution for 4 days for tissue cryoprotection. After that, the tissue was protected with plastic, quickly frozen in liquid nitrogen and stored in a freezer (~80°C) for subsequent sectioning [17]. The frozen tissue was cut into
30 μm semi-serial coronal sections along the dorsal hippocampus at a temperature of −25°C using a cryostat (Leica Biosystems, Nusslock, Germany). The dorsal hippocampus was collected and the sections were placed serially in ten well-plates using −2.56 to −4.52 mm stereotaxic coordinates in relation to the bregma [34], totaling six to eight 300 μm cuts per well-plate. Brain sections were stored at −20°C in cryoprotectant solution containing 30% ethylene glycol and 15% sucrose in 0.05 M phosphate buffered solution for subsequent processing [17, 41].

The free-floating method was used for immunohistochemical reactions with the IBA1 marker. The brain sections were initially washed with buffer A (0.1 M PBS, pH 7.4 with 0.5% Triton X-100) and incubated in 0.1 M citrate buffer, pH 6.0 in a water bath at 50°C for 30 min. The steps described above characterize an antigen retrieval step for this marker. After that, the sections were cooled to room temperature, washed with buffer A, incubated with 0.5% H2O2 solution in 0.1 M PBS for 30 min at room temperature and protected from light, washed again with buffer A and incubated with 2% bovine serum albumin (BSA) in buffer A (blocking buffer) at room temperature for 1 h. Subsequently, the plates containing the sections were incubated overnight at 4°C with the primary antibody diluted in anti-IBA1 goat polyclonal blocking buffer (1 : 500, Abcam, Cambridge, MA, USA) [17, 41].

The following day the sections were washed in buffer A and incubated with biotinylated secondary antibody at 4°C for 2 h, washed again in buffer A and incubated with ABC reagent (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) in 0.1 M PBS at room temperature for 2 h. After being washed with 0.1 M PBS, the reaction was revealed by incubating the sections with 3,3’-diaminobenzidine (DAB, Vector Laboratories, Burlingame, CA, USA) in 0.1 M PBS at room temperature. The sections were washed in 0.1 M PBS, mounted on gelatinized slides and air dried [17, 41].

Statistical analysis

The data are presented as mean ± standard error of the mean (SEM). The data were tested for a Gaussian distribution using the Kolmogorov-Smirnov test. Group differences were analyzed using one-way analysis of variance (ANOVA) with Bonferroni’s post-hoc test for parametric data or Kruskal Wallis (IBA-1 analysis in the CA1 region of the Hippocampus) with Dunn’s post-hoc test for non-parametric data. Values of p < 0.05 were considered statistically significant.

RESULTS

Open field test

The analysis of number of quadrants traveled by the animals (Fig. 1A) at day 27 after surgery showed no significant motor activity differences between groups. Thus, there were also no changes regarding motor capacity between groups. In addition, the OFT allowed the analysis of rearing behavior (Fig. 1B), when the animals stood on both hind legs. This analysis showed no significant exploratory capacity differences between groups, so all animals in this study had similar exploratory activities.

AZI formulation effects on animal cognition

Animal recognition memory was evaluated using the ORT (Fig. 2A) at day 28. The execution
protocol requires a 1 h interval between training and test sessions.

Animals in the STZ + saline group showed a statistically significant decrease in the discrimination index compared to the sham + saline group, showing that STZ-lesioned animals were unable to discriminate new and familiar objects. Animals in the STZ + AZ1 group showed a statistically significant increase in the discrimination index compared to the STZ + saline group, showing that AZ1 formulation was able to revert the memory loss caused by STZ. In addition, short-term spatial memory evaluated by spatial version of the Y-maze test (Fig. 2B) showed that the rats in the STZ + saline group spent a significantly decreased percentage of time exploring the new arm compared to the sham + saline group. The AZ1 formulation treatment significantly reversed cognitive impairment in STZ-lesioned rats in the STZ + AZ1 group.

**AZ1 formulation effects on neuroinflammation**

IBA1 immunoreactivity (Fig. 3) was used as a parameter to analyze the influence of AZ1 formulation treatment on glial reactivity and neuroinflammation. Our data showed increased IBA1 immunoreactivity in the three analyzed hippocampal regions (CA1, CA3, and DG - dentate gyrus) in the STZ + saline group compared to the sham + saline group (Fig. 4). Immunoreactivity to IBA1 reduction in CA1 and DG regions (Fig. 3A–C) showed that AZ1 formulation treatment significantly reduced glial activation in STZ-lesioned animals; however, it did not change the marking in the CA3 region (Fig. 3B) compared to sham + saline animals. The groups sham + saline and sham + AZ1 formulation showed no significant differences in IR to IBA1 in any hippocampal region.

**DISCUSSION**

Prolonged AZ1 formulation treatment reversed cognitive impairment in both the ORT and the Y-maze test and reduced neuroinflammation in CA1 and DG hippocampal regions in STZ-lesioned rats.

This neuroprotective effect of AZ1 formulation may be due to the combination of several lipids, such as caprylic and capric acid, which produce ketone bodies and are a source of brain energy, and the combination of DHA and phosphatidylserine, which restructure neuronal membranes and have an anti-inflammatory action. In addition, the presence of choline, acting on plasma phospholipid synthesis and structuring of cell membranes, vitamins and minerals which are extremely important for brain health.

An increased inflammatory process is associated with increased activation of microglia cells and astrocytes and progressive cognitive decline in humans [44, 45]. Postmortem studies in humans showed CA1 and CA3 hippocampal regions presenting decreased neuronal density in AD patients [46]. Thus, the results of the present study corroborate the neuroinflammation reported in other studies [16, 17, 41].

Our results show that the STZ-lesioned animals that received only saline solution presented the short-term object recognition and spatial memory deficits already mentioned in the literature, respectively by decreasing the discrimination index in the ORT [47, 48] and the time spent on the new arm in the Y-maze test [48]. In addition, STZ-related decreased spatial memory was already described in the Morris water maze test [49]. On the other hand, STZ-lesioned
Fig. 3. Effects of AZ1 formulation on microglial cells (IBA1 immunoreactivity) on STZ-ICV-injected animals. IBA1-IR significantly increased in CA1 region 30 days after surgery in STZ-ICV-injected rats. The treatment with AZ1 formulation at a dose of 1 g/kg reduced IBA-IR in hippocampal regions CA1 (A) and DG (C) but showed no significant results in CA3 region (B). The data are shown as mean ± SEM; n = 4–6 per group; *p < 0.05; **p < 0.01 versus sham + saline group; #p < 0.05 versus STZ + saline group (one-way ANOVA with Bonferroni’s post-hoc test on IBA1 immunoreactivity in the CA3 and GD regions, and Kruskal Wallis with Dunn’s post-hoc test for the hippocampus CA1 region).

animals treated with the enteral AZ1 formulation showed significant improvement in the evaluated parameters when compared to STZ + saline animals, indicating that AZ1 formulation components reversed spatial and object recognition memory impairment.

Spontaneous locomotion and exploratory behavior are important in all test groups, since changes in these parameters could decrease the animal’s performance in subsequent cognitive tests [17]. Performance comparison among the four experimental groups showed no significant noncognitive behavior differences, demonstrating that all animals had similar characteristics regarding exploratory capacity and locomotion.

Some experimental studies reported that phosphatidylserine increased interneuronal communication through increased cell membrane fluidity and attenuated acetylcholine reduction, in addition to improving performance on tasks that test short-term learning and memory skills [21]. In humans, phosphatidylserine increased glucose use and memory in information processing and ability to perform daily activities [50–56]. The incorporation of phosphatidylserine into human membranes depends on phosphatidylserine and DHA availability [21, 22].

The brain is vulnerable to lipid peroxidation, which decreases cell membrane fluidity and damages membrane proteins, contributing to membrane breakdown. Thus, in addition to the ideal combination with phosphatidylserine, DHA increases synapses and membrane fluidity, resulting in a more effective neurotransmitter function; besides inhibiting free radical production and reducing oxidative stress [25]. The use of omega-3 fatty acids has been reported in numerous AD animal models [57–60]. In the STZ-induced AD model, Pardeshi et al. [26] showed that DHA improved the efficacy of the pharmacological agent used in the study by increasing survival and neuronal memory due to decreased oxidative stress and inflammation.

As for caprylic and capric acid and the consequent formation of ketone bodies, a clinical study reported better cognitive performance evaluated by logical memory and coding tests in individuals diagnosed with probable mild to moderate AD after ingesting 20 g of caprylic and capric acid. In addition, cognitive test scores were correlated with plasma ketone body levels [61].

Some antioxidant nutrients present in the AZ1 formulation, such as zinc, selenium, magnesium, vitamins C and E, may be related to neuronal protection, since oxidative stress leads to DNA
damage, which in turn leads to neuronal apoptosis and consequent neurodegeneration [62, 63]. In addition, AD-related decreased antioxidant levels can be observed in brain regions of elderly people [64]. Furthermore, vitamin D3 treatment improved spatial learning and functional memory in STZ-lesioned animals [65]. Vitamin D can prevent cognitive impairment and dementia through neuronal protection [66].

It should be highlighted that the ICV administration of STZ (1 mg/kg) was able to cause memory deficits that could be related to the earlier stages of AD. This combination of components used in the nutritional AZ1 formulation, which had never been tested together, was able to reverse STZ-induced memory impairment.

However, a possible limitation of this study is that we did not evaluate neither the amyloid-β protein nor tau expression. According to Salkovic-Petrisic et al. [67], increased amyloid-β deposition in the wall of meningeal capillaries and cortical blood vessels was found in icv-STZ rats. Results suggest that cerebral amyloid angiopathy observed 6 and 9 months after the STZ-icv injection seems to be a continuation and progression of the amyloid pathology observed already 3 months following the STZ-icv treatment in this non-transgenic sAD animal model. In this line, Ravelli et al. [68] showed that icv injections of STZ increase amyloid-β expression in the hippocampus STZ and increased phosphorylation of tau in mice.
In addition, according to Chen and collaborators [69], the deposition of amyloid-β proteins in this model begin to be observed after 3 months of the ICV injection of STZ. In the present study, the evaluation of the prolonged administration of the AZ formulation was given to the subjects during 30 days. Maybe in our experimental design the deposition of tau and amyloid-β proteins might not be sufficient to obtain conclusive results. Future studies, some of them now in progress, could help to answer these questions.

In conclusion, the present study showed that prolonged supplementation with AZ1 formulation reversed STZ-induced cognitive impairment in addition to decreasing the progression of neuroinflammation in CA1 and DG of the hippocampus.

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CONFICT OF INTEREST

MABF Vital; TB Bassani and LC Souza declare no conflict of interest. ELR Moura was a collaborator on a grant supported by Prodiet.; H Santos is Scientific Specialist on Prodiet. APM Celes is Technical Director and Co-founder of Prodiet.

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