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INVOLVEMENT OF THE SPHINGOSINE KINASE-SPHINGOSINE 1 PHOSPHATE (SPHK/S1P) COUPLE IN COGNITIVE DECLINE ASSOCIATED WITH DIABETES IN WISTAR RATS

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ABSTRACT

sphingosine-1 /sphingosine phosphokinase (SPHK/S1P) couple has a strong neuroprotective potential, particularly in the functioning of hippocampal neurons. Furthermore, it is established that diabetes is associated with cognitive decline, following damage to the nervous tissue. The aim of this work was to determine the involvement of the cerebral SPHK/S1P couple is involved in the maintenance of cognitive functions in rats in a model of diabetes induced by alloxan monohydrate. **Materials and Methods**: Male Wistar rats were used. They were divided into three groups (group 1: control, group 2: diabetics rats treated with distilled water and group 3: diabetics treated with D-erythro-dihydrosphingosine, a sphingosine phosphate inhibitor. Diabetes was induced by intraperitoneal injection of alloxan monohydrate at a single dose of 150 mg/kg. After confirmation of diabetes, the animals were treated with distilled water, for groups 1 and 2, and D-erythro-dihydrosphingosine (DEDHS) for group 3, for 28 days. Five days before the end of the treatments, working memory and spatial memory were tested in the object recognition and radial maze tests. The animals were then anesthetized and the hippocampus extracted from the brains. The S1P and SPHK content in the hippocampus was measured by mRNA amplification by polymerase chain reaction, after nucleic acid extraction. **Results**: Diabetes did not induce a significant alteration in the animals' preference for the novel object. However, inhibition of the SPHK/S1P pair led to a progressive decrease in the animals' preference for the novel object ($p < 0.05$). Furthermore, the number of errors made by the animals was significantly higher in the diabetic groups. Diabetes induced a depletion of the expression of the SPHK/S1P pair in the hippocampus of the animals. **Conclusion**: Our study showed that diabetes was associated with impaired working memory and a significant decrease in mRNA expression in the hippocampus of the animals. The SPHK/SP1 pair seems to be involved in neuroprotection against diabetes-associated cognitive impairment and provides insight into the underlying mechanism.

KEYWORDS: memory; diabetes; sphingosine phosphate; rat.

INTRODUCTION

Diabetes mellitus is a common metabolic disease characterized by hyperglycemia due to defective insulin secretion and/or action. Chronic hyperglycemia is associated with dysfunction of various organs. Cognitive impairment is counted among the lateral complications of diabetes.[1,2,3] However, the mechanisms underlying the development of cognitive dysfunction in diabetic patients have not been fully elucidated.[4]

Cerebral complications of type 1 and type 2 diabetes (T2DM) can be defined as “diabetic encephalopathy”. Furthermore, T2DM is reported to be associated with a high risk of dementia and Alzheimer’s disease.[2, 5]

Other epidemiological evidence supports an association between diabetes and cognitive impairment.[6,7,8] Sphingolipids are important signaling molecules and regulate a host of cellular processes involved in neurodegeneration.[9] The important role of these lipids in determining the biophysical properties, topology and integrity of the membrane and many cell signaling processes including apoptosis and proliferation has been described by several authors.[10, 11] Recent studies report that bioactive sphingolipids such as ceramide-1 -

phosphate, sphingosine, sphinganine, sphinganine-1-phosphate (SA1P) and sphingosine-1-phosphate (S1P) are involved in neurodegeneration and brain aging. S1P has important neuroprotective properties and plays a critical role in neuronal excitability and transmission in the hippocampus.[12, 13]

The increasing prevalence of diabetes worldwide and its associated pathologies justify the need to understand the biological mechanisms involved. Under the hypothesis that the S1P/SPK couple could be involved in memory processes, several studies have evaluated its role in the physiology of the central nervous system.

In this study, we investigated the involvement of the sphingosine-1 /sphingosine phosphokinase couple in cognitive impairment in a rat model of diabetes induced by alloxan monohydrate.

MATERIALS AND METHODS

Animals and treatments

Male Wistar rats aged seven (07) to ten (10) weeks from the animal facility of the Faculty of Health Sciences were purchased used. They were housed in polystyrene cages and maintained under optimal temperature and humidity conditions (21 ± 1◦C and 55 ± 2% humidity) under a 12-h light/dark cycle and free access to food and water. The animals were acclimatized to laboratory conditions for 2 weeks before the start of the experiment. This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health in the United States.[14]

The animals were divided into three (03) groups composed of 05 rats each and treated as follows.
- group 1 received distilled water and served as a control group;
- group 2 composed of diabetic rats, having received distilled water;
- group 3 composed of diabetic rats treated with D-erythro-dihydrosphingosine (DEDHS).

The products (distilled water and DEDHS) were administered orally for 28 days.

Induction of diabetes

Diabetes was induced by a single intraperitoneal administration of alloxan monohydrate at a dose of 150 mg/kg body weight. The animals were housed in individual cages with free access to food and 5% glucose solution to prevent hypoglycemic shock.[15] Three days after alloxan monohydrate administration, diabetes was confirmed by measuring blood glucose levels using a glucometer (On Call PlusII). A drop of blood obtained by a slight incision at the tip of the tail was used to measure the blood glucose levels of the animals. Only rats with blood glucose levels greater than 180 mg/dl were selected for the experiment.[16]

Determination of animal weight

The selected animals were weighed using an electric scale at D_0, D_7, D_{14}, D_{21}, and D_{28}. This monitoring of weight change made it possible to validate, with blood sugar, the onset of diabetes in the animals.

Behavioral tests

Object recognition test

This test is based on the natural curiosity of animals for new objects. The technique described by [17] used. The device used consisted of an open cage with dimensions 60 cm L; 60 cm W; 30 cm H. The objects to be discriminated were a cylinder (dimensions) and a cube (dimension) and had no natural meaning for the animals. In order to exclude any olfactory traces left on the objects and in the arena, and thus the dependence of the rats' recognition ability on the olfactory cue, the objects and the interior of the device were wiped clean between each trial.

During the habituation phase, animals were placed in the arena, without objects, and free to explore the environment for 10 minutes, in order to reduce the anxiety-provoking aspect of the context.

The acquisition phase consisted of placing the animals in the arena for 10 minutes in the presence of two identical objects, called familiar objects.

Twenty-four hours after the acquisition phase, one of the two (familiar) objects was replaced by a novel object of a different shape. The animals were then placed in the box and free to explore this environment. For each rat, the time spent exploring the familiar object (T_F) and the novel object (T_N). The preference for the novel object was expressed as the percentage of time spent exploring the novel object compared to the total time spent exploring both objects (Rispoli et al., 2013; Ennaceur and Delacour, 1988; Antunes and Biala, 2012). Exploration was considered when the rat touched the object or brought its nose closer to the object, at a distance of less than 2 cm.

The radial labyrinth

Spatial memory was assessed in the 8-arm test. Each arm measured 36 x 38 x 16 cm and the octagonal central platform was 49 cm in diameter. A food pellet was placed at the distal end of each arm and allowed the reinforcement of the animal's behavior. The test was conducted in two phases: a training phase and a test phase. During the training phase, the food pellet was first placed in the center of the device and then at the ends of the arms. For each trial, the rat was placed in front of the same arm and left in the maze until it visited all 8 arms. After the seventh training session, the task (test phase) was identical to the regular trials: rats were placed in the center of the maze and had a maximum of 10 minutes to visit all eight arms in order to receive the food rewards placed at the end of each arm. An arm was counted as visited (corresponding to a success) if the rat crossed

two-thirds of the arm length, whether or not the arm was visited and food consumed. Errors were scored as entries into previously visited branches during the same session. Between trials, rats were returned to their usual cages.

Performance is scored by three measures of choice accuracy: number of correct choices in the first eight arm visits; choices where the first error was made; and total number of choices to complete the task.

Brain harvesting and seahorse isolation

Twenty-four hours after the behavioral tests, the animals were sacrificed by decapitation. The brain was then removed from the skull and the hippocampus was extracted from the latter, rinsed in a bath of phosphate-buffered saline solution. It was then stored in a sterile bottle at 80°C until molecular analyses.

Molecular analyses

Hippocampal samples were assayed for S1P1, SPHK1, and SPHK2 gene expression after DNA extraction using the ReliaPrep™ gDNA Tissue Kit (Promega) according to the manufacturer's instructions. DNA presence in the extracts was verified using the Qubit® 3.0 fluorometer.

Gene expression analysis was performed using RT-PCR technology (TaqMan). RT-PCR was performed on 1 ng of total DNA per reaction using the TaqMan sequence detector (Magnetic Induction Cycler, MIC model).

The primer sequences were determined from the above genes found in the NCBI Gene Bank database. The primer sequences used were as follows.
S1P1 (F- CTTGAGCGAGGCTGCTGT; R-GACGGTGGTGCAGAAGAGAA);
SPHK1 (F- GGTTCCTCCAGTTGGTGAGG; R-TTTTGCTCAACTTCGCCACG);
SPHK2 (F- GAGTATTACAAGACAGGCCAGC; R-CACGTGCATGGTTTTGTCGT).

The reaction volume was 25 µl, consisting of 12.5 µl of PCR buffer, 1.15 µl of Ppmix (probe and 25 mM dNTPs), 1 µl of enzyme (Taq polymerase), 0.5 µl of each primer and 9 µl of DNA. The amplification program was as follows: initial denaturation 3 minutes, followed by 40 cycles which alternated denaturation 30 seconds (94°C), annealing 8 seconds (94°C) and final extension 34 seconds (60°C).

Statistical analyses

Quantitative variables are presented as mean ± standard deviation and qualitative variables as numbers and percentages. Data were processed with Microsoft Excel 2016 software for database design and Graph Pad version 8 software (California, USA) for statistical analyses. The ANOVA test was used to compare groups. The *p value <0.05* was considered significant.

RESULTS

Evolution of body weight and blood sugar levels in animals

The diabetic state resulted in a significant decrease in the weight of the animals, compared to the control group (p = 0.03). Conversely, a strong increase in serum glucose concentration was observed in rats injected with alloxan monohydrate (*p = 0.0068*). DEDHS administration did not induce statistically significant changes in either weight gain or glucose concentration (*p > 0.05*). These results are presented in Figures 2 and 3.

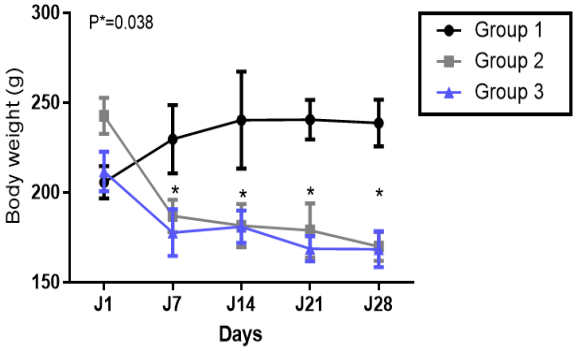


Figure 2: Evolution of body weight. Results are expressed as mean ± standard error. (D): day. (**): p =0.0360; n=5 animals per group. G roup 1 : control group; G roup 2 : untreated diabetic animals; G roup 3: diabetic animals treated with D-erythro-dihydrosphingosine (DEDHS).

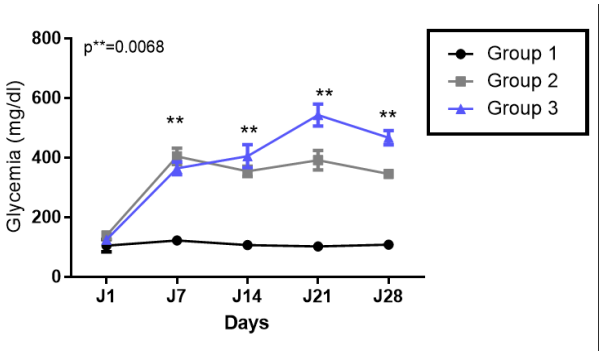


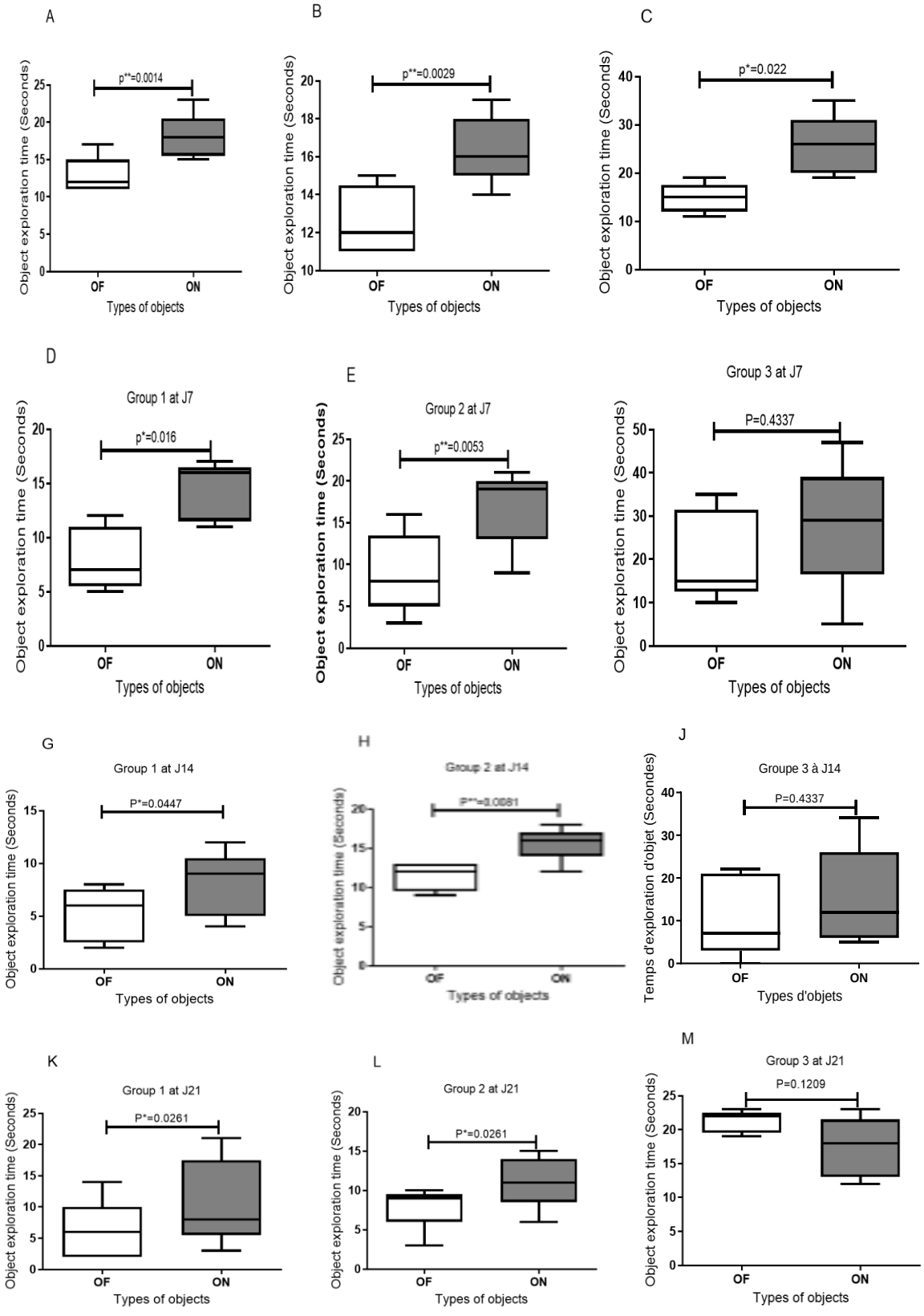
Figure 3: Evolution of mean glycemia in rats. Results are expressed as mean ± standard error. (J): day. (**): p=0.0068; n=5 animals per group. Group 1: control; Group 2: untreated diabetic animals; group 3: diabetic animals treated with D-erythro-dihydrosphingosine (DEDHS).

Effects on animal behavior

Object recognition test

Figure 4 shows the exploration time of the familiar object (FO) and the new object (NO) in the different groups, at D1, D7, D14, D21 and D28. The analysis of the results showed that the exploration time of the new object by the animals was greater than that of the familiar object, in groups 1 and 2. However, the animals of group 3 did not present any significant difference in the exploration times of the familiar object and the new

object, from D7. These results suggest on the one hand that there is no alteration of memory, evaluated by the object recognition test, in diabetes induced by alloxan monohydrate and on the other hand, that inhibition of SPK1 and 2 could be responsible for the observed memory alterations.



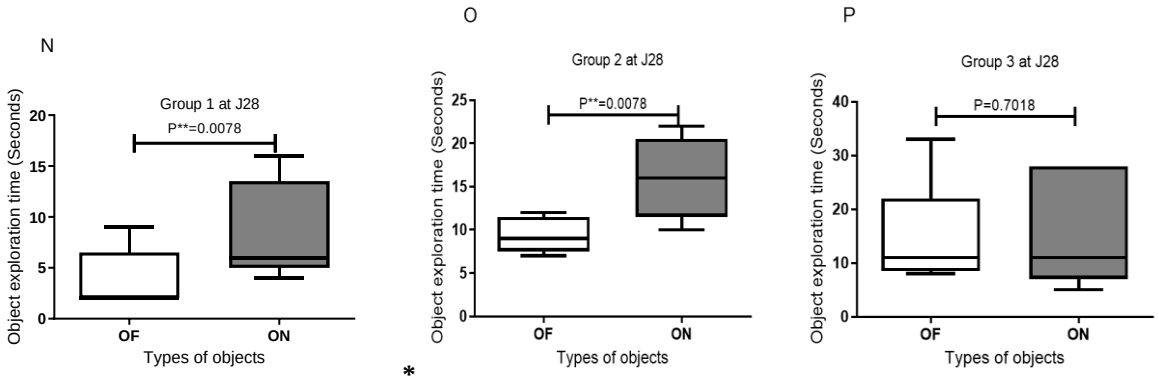


Figure 4: Effect on object recognition. Results are expressed as mean ± standard error. (J): day. n=5 animals per group. Group 1: control; Group 2 : untreated diabetic animals; Group 3: diabetic animals treated with D-erythro-dihydrosphingosine (DEDHS). ON: novel object and OF: familiar object.

The radial labyrinth

The number of errors made by animals in groups 2 and 3 were significantly higher compared to animals in the control group, from D14 (p = 0.0039). No difference in the number of errors made by animals in groups 2 and 3. These results are presented in Figure 5. These data suggest that diabetes impairs working memory and this impairment is not corrected by D-erythro-dihydrosphingosine (inhibition of the SPHK/SP1 couple).

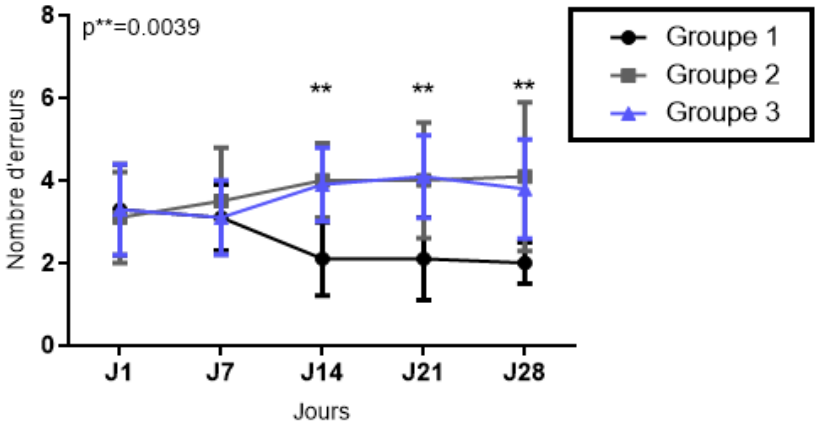


Figure 5: Radial maze task. Mean number of errors for each session. Results are expressed as mean ± standard error. (D): day. n=5 animals per group; (**): p=0.0039 G roup 1: control; G roup 2: untreated diabetic animals; G roup 3: diabetic animals treated with D-erythro-dihydrosphingosine (DEDHS).

Expression of S1P, SPK1 and SPK2 mRNA

Our data showed a significant decrease in the expression of SP1, SPK1 and SPK2 enzymes mRNA in the hippocampus of animals in groups 2 and 3 compared to animals in the control group (p= 0.0008, p*=0.04 and p*=0.027, respectively) These data suggest that diabetes inhibits the expression of S1P, SPK1 and SPK2.

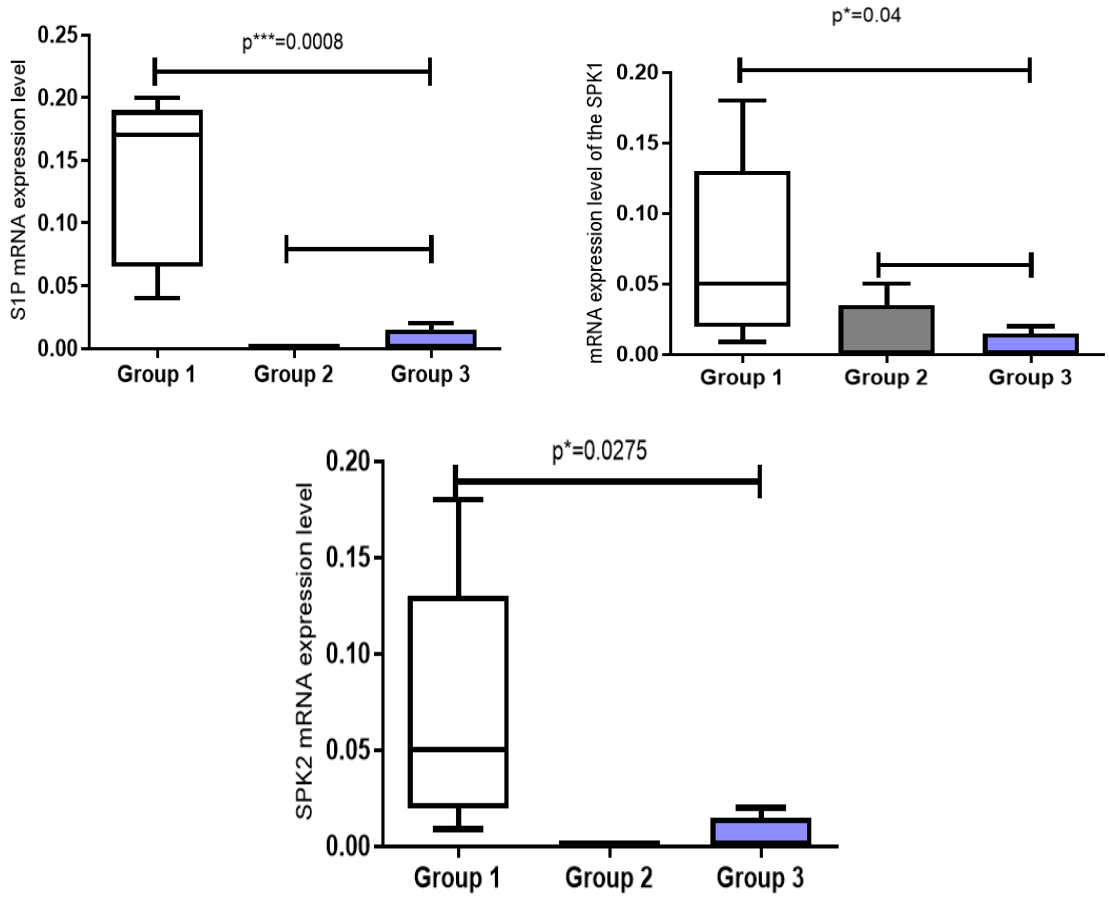


Figure 6: Expression level of SP1, SPK1 and SPK2 enzymes mRNA in the hippocampus of animals. Results are expressed as mean ± standard error. N=5 rats per group. Group 1: control; Group 2: untreated diabetic animals; Group 3: diabetic animals treated with D-erythro-dihydrosphingosine (DEDHS).

DISCUSSION

Several studies highlight the important role of sphingolipids in the physiology and pathophysiology of the central nervous system.[18] However, the literature reports little data on the involvement of S1P sphingolipids and SPHK enzymes in cognitive deficits associated with diabetes. The aim of this study was therefore to evaluate the involvement of the SP/SPK couple in cognitive decline associated with diabetes in rodents.

In this study, diabetes was induced by injection of a single dose of alloxan monohydrate (150 mg/kg). Alloxan is a commonly used substance to induce experimental diabetes. It causes necrosis of pancreatic cells.[19] This preferential destruction of beta cells is manifested by hyperglycemia and weight loss. In this study, diabetic animals exhibited weight loss that began on day 7. The weight loss is due to insulin deficiency that leads to decreased absorption of amino acids by tissues, with consequent reduction in protein synthesis and increased mobilization of body fat.[20] Type 2 diabetes (T2DM) are frequently associated with reduced performance in multiple domains of cognitive function, with evidence of abnormal brain structure in functional magnetic resonance imaging (fMRI), reasoning speed, mental flexibility, and memory.[21, 22 23] Thus, there is increasing evidence that diabetes predisposes to cognitive decline leading to dementia in animal models and in humans with type 1 diabetes.[24, 25] Diabetic encephalopathy is a recognized complication of untreated diabetes leading to progressive cognitive impairment, accompanied by changes in brain functions, such as cognitive performance.[26] The malfunction in each interconnecting pathway ultimately leads to discordance in metabolic signaling, although the duration of diabetes and glycemic control could impact the type and severity of cognitive impairment deficiency.

In this study, cognitive performance was assessed in the object recognition and radial labyrinth tests. Analysis of the results revealed no significant differences between control animals and diabetic animals not treated with the S1P inhibitor, in the object recognition test. Diabetic animals not treated with the SP inhibitor retained the preference for the novel object. This observation suggests that diabetes would not affect working memory and seems to contradict other authors.[27] However, diabetic animals treated with the inhibitor, showed a progressive decrease in preference for the novel object. These results imply that the S1P/SPHK couple would contribute to neuroprotection against diabetes-related

cognitive failures. It is also unlikely that the absence of effect on object recognition tasks simply reflects insensitivity to the test. Previous studies using this paradigm have shown that performance is systematically affected by delays and by systematic changes in test similarity.[15, 28]

Sphingosine 1-phosphate (S1P) are bioactive lipids that mediate a diverse range of effects through activation of cognate receptors, S1P1-S1P5. Cumulatively, there is significant preclinical evidence implicating critical roles for this pathway in the regulatory processes that drive cerebrovascular disease and vascular dementia, both part of the continuum of cognitive impairment.[29] This is supported by clinical studies that have identified correlations between S1P alterations and cognitive deficits.

Molecular analyses of animal hippocampi revealed a significant decrease in S1P, SPK1, and SPK2 mRNA expression in diabetic animals. Therefore, the net effect produced by S1P represents the cumulative contributions of S1P receptors acting additively, synergistically, or antagonistically on neural, vascular, and immune cells in the brain. Furthermore, the level of SP1 mRNA, although low, would maintain a residual expression of SP1 sphingolipids, which would be responsible for the neuroprotection observed in the object recognition test.

CONCLUSION

Our study showed that diabetes was associated with impaired working memory, which was reflected by an increase in the number of errors made by animals in the 8-arm maze test, and a significant decrease in mRNA expression in the hippocampus of animals. Furthermore, the SPHK/SP1 couple seems to be involved in neuroprotection against diabetes-associated cognitive impairments and provides insight into the underlying mechanism.

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