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Research article

An evaluation of aversive memory and hippocampal oxidative status in streptozotocin-induced diabetic rats treated with resveratrol

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HIGHLIGHTS

- Diabetic rats exhibited normal freezing response in contextual fear conditioning.
- Hippocampal oxidative status was unaltered in diabetic rats.
- Resveratrol oral treatment had no significant effects in healthy or diabetic rats.

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ABSTRACT

The present study evaluated the effects of streptozotocin (STZ)-induced diabetes on aversive memory, free radical content and enzymatic antioxidant activity in the hippocampus of adult *Wistar* rats submitted to oral treatment with resveratrol. Animals were divided into eight groups: non-diabetic rats treated with saline (ND SAL), non-diabetic rats treated with resveratrol at a dose 5 mg/kg (ND RSV 5), non-diabetic rats treated with resveratrol at a dose 10 mg/kg (ND RSV 10), non-diabetic rats treated with resveratrol at a dose 20 mg/kg (ND RSV 20), diabetic rats treated with saline (D SAL), diabetic rats treated with resveratrol at a dose 5 mg/kg (D RSV 5), diabetic rats treated with resveratrol at a dose 10 mg/kg (D RSV 10) and diabetic rats treated with resveratrol at a dose 20 mg/kg (D RSV 20). The animals received oral gavage for 35 days. The contextual fear conditioning task was performed to evaluate aversive-based learning and memory. The oxidative status was evaluated in the hippocampus, by measuring the free radical content – using a 2′,7′-dichlorofluorescein diacetate probe – and enzymatic antioxidant activities, such as superoxide dismutase and glutathione peroxidase. Our main behavioral results demonstrated that rats from the D RSV 10 and D RSV 20 groups showed an increase in freezing behavior when compared, respectively, to the ND RSV 10 ($p < 0.01$) and ND RSV 20 ($p < 0.05$). Oxidative stress parameters remained unchanged in the hippocampus of all the experimental groups. In contrast to previous experimental findings, our study was unable to detect either cognitive impairments or oxidative stress in the hippocampus

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of the diabetic rats. We suggest additional long-term investigations be conducted into the temporal pattern of STZ-induced diabetic disruption in memory and hippocampal oxidative status, as well as the effects of resveratrol on these parameters, in a time and dose-dependent manner.

1. Introduction

Diabetes mellitus comprises a group of metabolic disorders characterized by hyperglycemia resulting from defects in insulin secretion and/or insulin insensitivity [1]. Recent clinical studies have confirmed that diabetes is associated with cortical atrophy, white matter lesions and the development of cognitive dysfunctions, which include impaired attention, intelligence, psychomotor speed and psychomotor efficiency [2,3]. However, learning and memory deficits were not observed in all clinical studies [4–6]. Therefore, it has been suggested that such clinically relevant cognitive deficits might be related to the beginning of diabetes in two specific periods in life: during brain development in childhood, and when the brain undergoes neurodegenerative changes associated with ageing [7].

Despite differences between clinical data, deficient insulin signaling in the brain and hyperglycemia are considered potential contributors to the development of neurological complications in diabetes [3,8,9]. It has been reported that insulin participates in the regulation of apoptosis and oxidative status in the central nervous system (CNS), which could explain the link between disruption in insulin activity and neuronal and oligodendroglial degeneration in the diabetic brain [8,10]. Hyperglycemia could raise free radical formation in the CNS through different mechanisms, including increased glycolysis, activation of the polyol pathway and increased formation of advanced glycation end-products (AGEs) [11,12]. Moreover, reduced antioxidant activity has been observed in diabetic rodents, which together with the above-mentioned evidence may contribute to oxidative stress and to the development of cognitive dysfunctions in diabetes [3,8,13]. Nevertheless, some studies present contrasting findings, with no significant indications of learning and memory deficits, nor significant alterations in some parameters related to oxidative stress in the diabetic rodent brain, especially in the hippocampus [14–16].

Resveratrol, a polyphenolic compound found in peanuts, some berries, grapes and wine, is known to have beneficial health effects [17]. This compound presents antioxidant activities in the CNS, demonstrated by the ability to scavenge free radicals and up-regulate antioxidant enzymes [18]. Additionally, studies have suggested resveratrol produces benefits in the CNS of diabetic rodents, protecting them from memory impairment, reducing the release of pro-inflammatory factors and reestablishing normal antioxidant enzyme levels [19,20].

The aim of this study was to verify the effects of streptozotocin (STZ)-induced diabetes on aversive memory, free radical content and enzymatic antioxidant activity in the hippocampus of adult *Wistar* rats, and evaluate the influence of oral treatment with resveratrol on these parameters.

2. Material and methods

2.1. Animals

All procedures were conducted in accordance with the University guidelines, and were previously approved by the animal ethics committee of the University. Sixty-two male *Wistar* rats obtained from the *Centro de Reprodução e Experimentação de Animais de Laboratório* (CREAL, *Universidade Federal do Rio Grande do Sul* – UFRGS), aged 12 weeks at the start of the experiment, were used. They were maintained under standard laboratory conditions, with free access to rat chow and water and a 12:12 light/dark cycle (lights on from 08:00 to 20:00 h). The animals were randomly divided into eight groups, as follows: non-diabetic rats treated with saline (ND SAL), non-diabetic rats treated with resveratrol at a dose of 5 mg/kg body weight (ND RSV 5), non-diabetic rats treated with resveratrol at a dose of 10 mg/kg body weight (ND RSV 10), non-diabetic rats treated with resveratrol at a dose of 20 mg/kg body weight (ND RSV 20), diabetic rats treated with saline (D SAL), diabetic rats treated with resveratrol at a dose of 5 mg/kg body weight (D RSV 5), diabetic rats treated with resveratrol at a dose of 10 mg/kg body weight (D RSV 10) and diabetic rats treated with resveratrol at a dose of 20 mg/kg body weight (D RSV 20). For the behavioral task, 7 to 8 rats per group were analyzed. For the biochemical analyses, 5 to 6 rats per group were used. A timeline with our experimental design can be seen in Fig. 1.

2.2. Diabetes induction

After an overnight fasting period, diabetes was induced by a single intraperitoneal (i.p.) injection of STZ (65 mg/kg of body weight) diluted in 0.1 M sodium-citrate buffer, pH 4.5. The non-diabetic rats received an equivalent amount of sodium-citrate buffer. 72 h after i.p. injections, blood glucose levels were measured in blood collected from the rat tail using a portable glucometer (On Call Plus, ACON Laboratories, USA). Only animals with blood glucose levels >300 mg/dL and symptoms of polyuria and polydipsia were considered diabetic and selected for the present study. During the experiment, the blood glucose levels of all the animals were verified at four moments: D1 (before i.p. injections of STZ and/or vehicle), D4 (72 h after i.p. injections), D30 and D64.

2.3. Oral gavage

From D30 to D64, oral treatment was provided to all groups once a day, between 10:00 and 11:00 a.m., totaling 35 days of treatment. Resveratrol was freshly dispersed in 0.9% saline solution and promptly administered via oral gavage to animals belonging to the ND RSV groups and the D RSV groups, in their respective doses, based on previous studies [14,21]. Animals from the ND SAL and D SAL groups received equal volumes of 0.9% saline solution alone. Resveratrol was stored at 5 °C in an amber flask, protected from light. The body weights of all the animals were verified on D1, D4 and D30 and, in order to better control the resveratrol dose, from D30 to D64, they were verified twice a week (on Mondays and Thursdays).

2.4. Contextual fear conditioning (CFC)

At D63, each rat was placed in a chamber (25.0 × 25.0 cm grid of parallel 0.1 cm caliber stainless steel bars, spaced 1.0 cm apart). In the training session, rats were placed in the chamber during 3 min for habituation, and after received two 2-s foot shocks of 0.7 mA, separated by an interval of 30 s. After the last foot shock, animals were kept in the conditioning chamber an additional minute, and

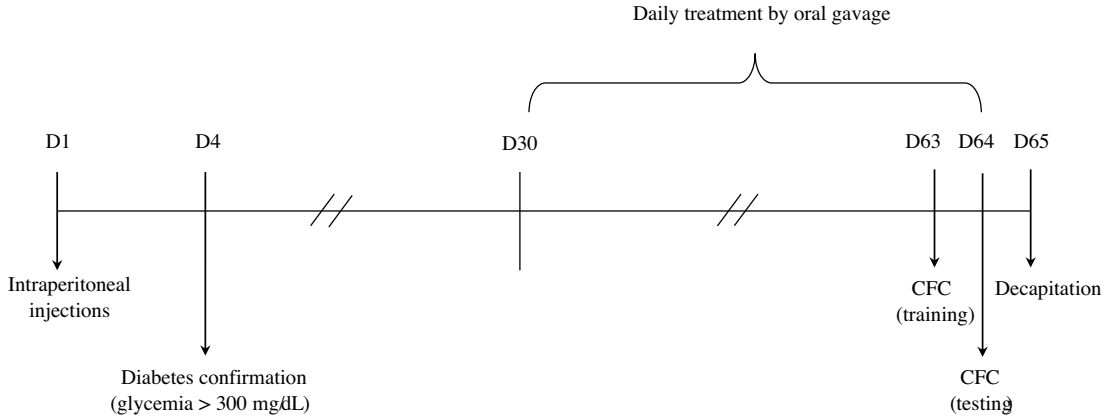

Fig. 1. Timeline of the experimental design. D = day, CFC = contextual fear conditioning.

then returned to their home cages. At D64 (24 h later), the rats were placed in the same context (without foot-shocks) for the testing session, and the freezing behavior of the animals were clocked during 5 min [22].

2.5. Biochemical analysis

At D65, the rats were killed by decapitation. Brains were removed from the skulls, hippocampi of animals were rapidly dissected out on ice and frozen in liquid nitrogen. They were stored at −80 °C until the biochemical measurements were taken. On the day of analysis, the hippocampi were homogenized in ice-cold phosphate buffer (0.02 M, pH 7.4) containing EDTA (0.002 M) and phenyl-methylsulfonyl fluoride (PMSF, 0.1 M) in a Teflon-glass homogenizer. Afterward, the homogenate was centrifuged at 1000 × *g* for 10 min at 4 °C, and the supernatant was used.

2.5.1. Reactive species levels

Reactive species levels were quantified using 2′,7′-dichlorofluorescein diacetate (DCFH-DA) as a probe [23]. The oxidized fluorescent derivative (DCF) formation was monitored at excitation (488 nm) and emission (525 nm) wavelengths using fluorescence spectrophotometer. The reactive species content was measured using a DCF standard curve and results were expressed as nmol DCF formed per mg of protein. All procedures were performed in the dark, while the blanks containing DCFH-DA (no homogenate) were processed for auto fluorescence measurement [24,25].

2.5.2. Superoxide dismutase (SOD) activity

The SOD activity was evaluated using a specific kit following the manufacturer's instructions (RANSOD kit, Randox Labs, USA). This method employs xanthine and xanthine oxidase to generate a superoxide anion radical that reacts with 2-(4-iodophenyl)-3-(4-nitrophenol)-5 phenyltetrazolium chloride (I.N.T.) to form a red formazan dye, which is measured at 37 °C using a spectrophotometer reader (505 nm). The inhibition of chromogen production is proportional to the SOD sample activity and the data were expressed as unit of SOD per mg of protein.

2.5.3. Glutathione peroxidase (GPx) activity

GPx activity was determined according to Wendel (1981) [26]. The reaction was carried out at 25 °C in 600 μl of solution containing 100 mM pH 7.7 potassium phosphate buffer, 1 mM EDTA, 0.4 mM sodium azide, 2 mM GSH, 0.1 mM NADPH, 0.62 U of GSH reductase. The activity of selenium-dependent GPx was measured taking *tert*-butyl-hydroperoxide as the substrate at 340 nm. The contribution of spontaneous NADPH oxidation was subtracted from the overall reaction rate. GPx activity was expressed as nmol NADPH oxidized per minute per mg of protein.

2.5.4. Protein determination

The total protein content of the tissue homogenates was measured by means of the Coomassie blue method using bovine serum albumin as standard, according to [27].

2.6. Data analysis

Body weight and blood glucose data were analyzed using repeated measures analysis of variance (ANOVA). The CFC and biochemical data were analyzed using two-way ANOVA. The Tukey's post-hoc test was used in these two ANOVAs. The results are shown as mean ± standard error of the mean (SEM). In all the statistical tests, significance was set at $p < 0.05$.

3. Results

3.1. Body weight and blood glucose

On day 1 (D1), there was no significant difference in body weight between the groups. However, on D4, the diabetic groups D SAL, D RSV 5, D RSV 10 and D RSV 20 presented a significant weight loss compared to the non-diabetic groups ND SAL, ND RSV 5, ND RSV 10 and ND RSV 20 ($p < 0.001$). The body weight of the diabetic groups remained significantly lower in relation to the non-diabetic rats on D30 and D64 ($p < 0.001$) (Table 1).

Analysis of the glycaemia data showed no differences in blood glucose levels between the groups on D1. However, from D4 onward, the blood glucose levels of rats from the diabetic groups D SAL, D RSV 5, D RSV 10 and D RSV 20 were significantly increased when compared to the non-diabetic groups ND SAL, ND RSV 5, ND RSV 10 and ND RSV 20 ($p < 0.001$) (Table 1).

3.2. Contextual fear conditioning (CFC)

Baseline freezing behavior was not measured during the habituation period in the training session. Fig. 2 shows the freezing behavior exhibited during the test session, held 24 h after the training session. No significant differences in the percentage of time in the freezing behavior were observed between the ND SAL and D SAL groups ($p > 0.05$). Our main behavioral results demonstrated that rats from the D RSV 10 and D RSV 20 groups showed an increase in freezing behavior when compared, respectively, to the ND RSV 10 ($p < 0.01$) and ND RSV 20 ($p < 0.05$).

Table 1
Body weight and blood glucose levels.

Groups	D1		D4		D30		D64	
	Weight (g)	Glycemia (mg/dL)	Weight (g)	Glycemia (mg/dL)	Weight (g)	Glycemia (mg/dL)	Weight (g)	Glycemia (mg/dL)
ND SAL	366.00 ± 9.76	91.62 ± 5.27	383.25 ± 11.92	86.00 ± 5.54	409.25 ± 13.95	86.25 ± 2.89	455.25 ± 34.66	85.63 ± 2.59
ND RSV 5	382.00 ± 13.05	87.25 ± 4.66	396.75 ± 12.84	91.88 ± 3.47	433.25 ± 16.57	88.25 ± 2.43	471.75 ± 15.95	80.75 ± 3.00
NDM RSV 10	372.75 ± 7.93	87.37 ± 3.16	393.75 ± 8.82	92.63 ± 3.54	426.50 ± 13.49	85.75 ± 3.49	471.75 ± 14.17	89.63 ± 5.82
ND RSV 20	388.00 ± 4.93	88.75 ± 2.70	403.00 ± 7.75	92.13 ± 2.93	418.00 ± 14.88	88.50 ± 4.22	475.00 ± 10.71	89.25 ± 4.72
D SAL	369.14 ± 7.96	81.00 ± 3.18	329.71 ± 11.80***	367.29 ± 14.13***	301.71 ± 13.45***	469.71 ± 25.40***	294.28 ± 11.77***	434.14 ± 20.48***
D RSV 5	393.75 ± 9.18	82.25 ± 3.04	345.75 ± 9.91***	351.13 ± 23.52***	317.25 ± 9.43***	498.13 ± 26.79***	311.50 ± 16.43***	513.88 ± 27.12***
D RSV 10	372.50 ± 9.16	87.88 ± 3.18	337.75 ± 9.51***	360.63 ± 18.07***	310.50 ± 9.71***	458.13 ± 32.15***	297.25 ± 12.23***	517.13 ± 22.07***
D RSV 20	375.71 ± 11.04	82.29 ± 3.27	329.14 ± 12.43***	332.71 ± 8.13***	300.57 ± 12.86***	428.29 ± 28.76***	288.85 ± 15.39***	474.00 ± 15.71***

ND SAL: non-diabetic rats treated with saline, ND RSV 5: non-diabetic rats treated with resveratrol at a dose of 5 mg/kg, ND RSV 10: non-diabetic rats treated with resveratrol at a dose of 10 mg/kg, ND RSV 20: non-diabetic rats treated with resveratrol at a dose of 20 mg/kg, D SAL: diabetic rats treated with saline, D RSV 5: diabetic rats treated with resveratrol at a dose of 5 mg/kg, D RSV 10: diabetic rats treated with resveratrol at a dose of 10 mg/kg, D RSV 20: diabetic rats treated with resveratrol at a dose of 20 mg/kg. D1: day 1, D4: day 4, D30: day 30, D64: day 64.
*** Corresponds to $p < 0.001$ compared to ND SAL, ND RSV 5, ND RSV 10 and ND RSV 20 groups.

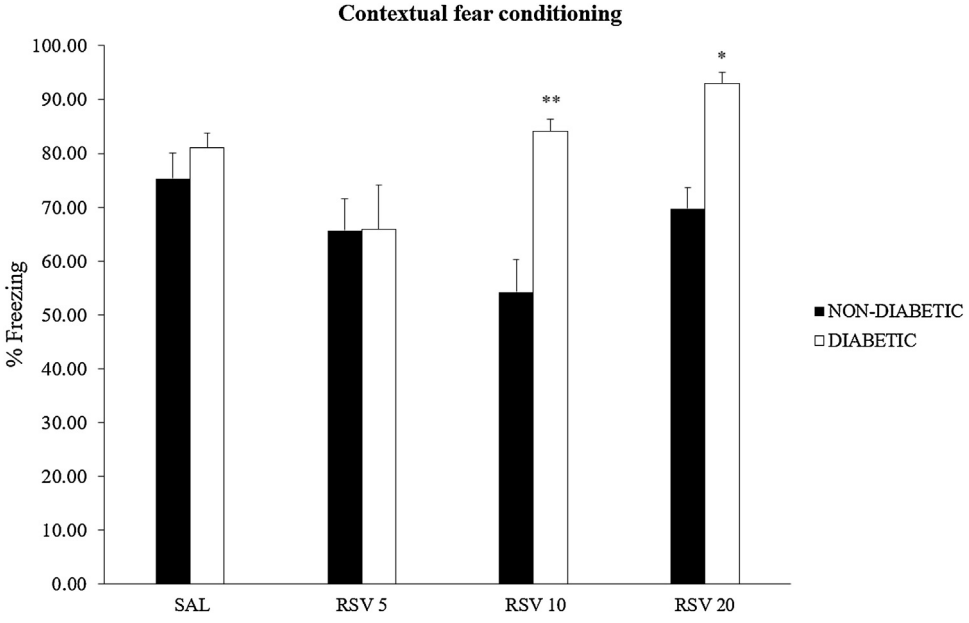

Fig. 2. Percentage (%) of freezing behavior measured in the test session. ** corresponds to $p > 0.01$ compared to the ND RSV 10, and * corresponds to $p > 0.05$ compared to the ND RSV 20. Data are mean ± SEM.

3.3. *Oxidative stress parameters*

There were no significant differences in the hippocampal free radical content ($p = 0.20$), SOD activity ($p = 0.59$) or GPx activity ($p = 0.74$) between the experimental groups (Fig. 3).

4. Discussion

Experimental STZ-induced diabetes is a widely used method for analysis of CNS dysfunctions, caused by impaired insulin signaling and hyperglycemia [3,28]. STZ-diabetic rodents are hypoinsulinemic, but do not require insulin treatment to survive [28]. The consequent high blood glucose levels lead to marked polyuria, polydipsia, and weight loss. In the present study, all these features were observed in the diabetic rats, and treatment with resveratrol was unable to influence the blood glucose levels or body weight of the diabetic and non-diabetic rats, as previous studies have shown [14,19,21].

In spite of the endocrine and metabolic changes caused by STZ-induced diabetes, our experimental design demonstrated that diabetes did not affect the conditioned response in the CFC task or hippocampal oxidative status in adult *Wistar* rats.

The CFC task is employed to evaluate fear/emotional learning and memory in rats, which are hippocampus-dependent memory processes [29]. We observed no significant differences in aversive memory assessed approximately 9 weeks after experimental diabetes induction. While studies suggest that fear learning and memory deficits are observed in diabetic rodents submitted to contextual fear tasks [30–32], it is important to note that some studies have failed to replicate these behavioral findings in diabetic rodents evaluated in CFC or spatial memory tasks [15,16,33]. These contrasting findings could be explained by differences in the experimental design used in each study, such as: the diabetic model selected (diabetes induction by STZ or other diabetogenic drugs, by specific diet, or the choice of transgenic rodent model); the route of administration and dose of the diabetogenic drug; age at the time of diabetes induction; the length of time the animal has diabetes prior to the behavioral analysis; the memory task employed and protocol characteristics.

In comparison to our study, the most similar paper is from Calgaroto et al. [31]. In that study, experimental diabetes was also induced by STZ injection in *Wistar* rats, and the percentage of freezing behavior was measured in the CFC task. It is very important to note that Calgaroto used three 0.4 mA foot shocks (1 s each) while, in our study, we used two 0.7 mA foot shocks (2 s each). These dif-

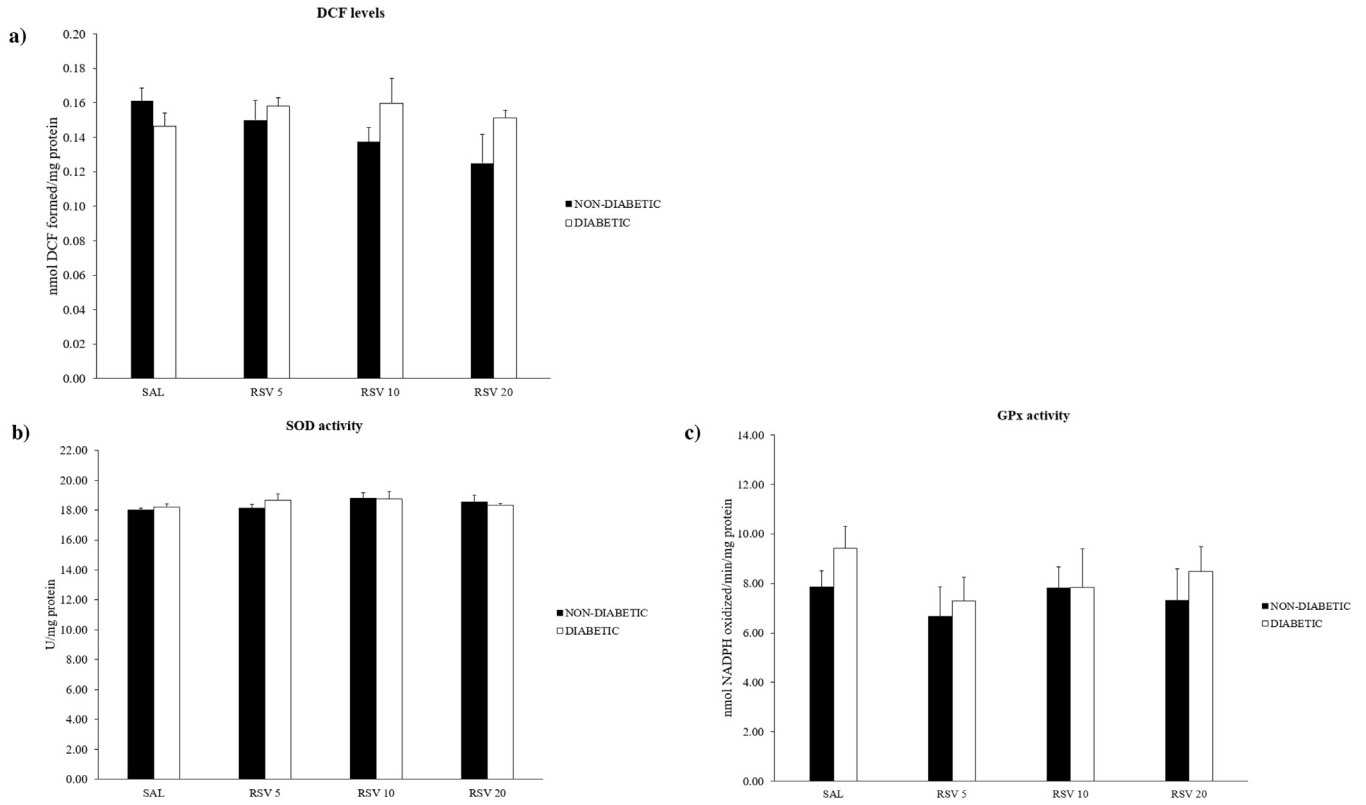

Fig. 3. Measurement of free radical content using DCFH-DA as a probe (a), and enzymatic antioxidant activity of SOD (b) and GPx (c) in the hippocampi of rats from all experimental groups. Data are mean ± SEM.

ferences result in a clear variation in the percentage of freezing. Calgaroto observed freezing behavior in almost 40% of the time in diabetic animals, while in control animals the percentage was almost 50%. By contrast, in our study the freezing behavior lasted between 70 and 80% of the time in diabetic and non-diabetic (non-treated with resveratrol) rats, indicating that our protocol is more intense, and possibly generated a stronger memory. It is probable that diabetes is able to impair fear-based learning and memory related to a mild stimulus (i.e. lower amperage shock) as produced by the Calgaroto protocol, but unable to impair fear memory related to strong stimulus (i.e. higher amperage shock).

Additionally, our main relevant results revealed that resveratrol oral treatment, at doses of 10 and 20 mg/kg, was able to improve aversive memory in diabetic rats, but only when compared to the respective non-diabetic groups, treated with the same doses of resveratrol. These findings suggest that resveratrol exerts a positive influence on hippocampal plasticity, which is probably more pronounced in diabetic rodents than in healthy animals. To our knowledge, this is the first study to analyze the effects of the oral consumption of resveratrol in diabetic rats submitted to a CFC test. For a better understanding of our results, the development of more studies are necessary, focusing on the detailed circumstances in which resveratrol could produce beneficial effects in healthy and diabetic rodents submitted to CFC and other hippocampal-dependent tasks.

In line with our behavioral findings, the present study found that oxidative status in the hippocampus, as assessed by determination of the free radical content and SOD and GPx activities, was unaffected in rats approximately 9 weeks after STZ-induced diabetes. Our results are in contrast with experimental evidence that suggests hyperglycemia and deficient insulin signaling in the brain contribute to disruption in the balance between pro-oxidant and antioxidant cascades, thus promoting oxidative damage and impaired hippocampal plasticity [3,9]. It is well known that, during hyperglycemia, glucose is diverted to metabolic pathways that may result in neurotoxicity in different brain regions, including the hippocampus [2,8]. One of which is the polyol pathway, which compromises the glutathione cycle by consuming the proton donor NADPH, thus decreasing the capacity of the GPx enzyme to metabolize hydrogen peroxide (H_2O_2) to water (H_2O), and increasing the production of highly reactive hydroxyl radical ($^{\bullet}OH$), through the passage of H_2O_2 into the Fenton reaction. In addition, with the flow of glucose through the tricarboxylic acid cycle, superoxide is produced in the mitochondrial electron-transport chain and the SOD enzyme catalyzes the dismutation of radical superoxide in H_2O_2 and H_20 [11]. All these events can lead to oxidative stress, a situation where oxidants prevail over antioxidants, leading to a disruption of redox signaling and control and/or molecular damage [34]. Also, hyperglycemia and insulin deficits increase the accumulation of AGEs, generated by the non-enzymatic reaction between sugars and amino groups. AGEs are able to irreversibly change the properties of different biological structures and bind to their receptors (RAGEs), promoting inflammatory responses via up-regulation of RAGEs and activation of the nuclear factor kappa B (NF-kB) in the hippocampus. The activation of this transcription factor leads to the activation of pro-inflammatory and pro-apoptotic signaling sequences, increasing ROS production and further damaging vascular endothelial cells, neurons and glial cells [8,9,35].

On the other hand, there are some studies in accordance with our findings, which have failed to demonstrate alterations in oxidative stress markers – more specifically, lipid peroxidation – and enzymatic antioxidant activity – including SOD, CAT and GPx activities – in the hippocampus of diabetic rats [14,36], probably because hyperglycemia modulates these processes in a time and region-specific manner in the rat hippocampus [37]. These could be interesting topics for future investigations.

Furthermore, resveratrol oral consumption did not have any significant effect on the free radical content, SOD or GPx activities in the hippocampus of any of the experimental groups. Venturini et al. (2010) showed that oral daily treatment with resveratrol at a dose of 20 mg/kg during three weeks significantly decreased the lipid peroxidation in the hippocampus of STZ-induced diabetic rats [14]. However, similarly to the present study, they observed no significant differences in enzymatic antioxidant activity in the hippocampus of diabetic rats treated with resveratrol, in comparison with all the other experimental groups [14]. Resveratrol has well-known health promoting effects supported by several mechanisms, including modulation of cellular redox status in the CNS [18,38]. The neuroprotective mechanisms initiated by resveratrol may be dependent on, and more pronounced after, significant disruptions in oxidative and inflammatory status of neural cells. This could explain the absence of resveratrol effects in the present study, since the hippocampal oxidative status was unaltered in our experimental groups. In order to provide more accurate information, we suggest the development of additional studies focusing on long-term investigations into the temporal pattern of STZ-induced diabetic disruption in memory and hippocampal oxidative status, and the effects of the daily intake of resveratrol on these parameters, in a time and dose-dependent manner.

5. Conclusions

STZ-induced diabetes during 9 weeks has no effects on aversive memory or oxidative status in the hippocampus of adult rats. Resveratrol oral consumption was unable to promote significant changes in these parameters, both in healthy and diabetic animals.

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