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Effect of Resveratrol on Behavioral Performance of Streptozotocin-induced Diabetic Mice in Anxiety Tests

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Abstract: The aim of this study was to evaluate with anxiety tests the effect of resveratrol (RSV) on streptozotocin (STZ)-induced diabetic mouse behavioral performance at the second and fourth week of treatment. Confirmed diabetic mice (>250 mg/dl of glucose in blood after STZ injection) were treated with RSV (RDM, n=12) or control treated (DM, n=12) for 4 weeks. DM and RDM were tested in the Open Field Test (OFT) and Elevated Plus Maze (EPM). In the second week of RSV treatment, a higher grooming frequency ($P<0.05$) and a lower defecation and rearing frequency ($P<0.05$) were detected in the OFT in the RDM group compared with the DM. There was a higher grooming frequency ($P<0.05$) and higher percentage of entries in open arms ($P<0.05$) in the RDM group than in the DM group in the EPM. However, in the fourth week of RSV treatment, the only effect observed was a higher grooming frequency in the RDM group than in the DM group ($P<0.05$) in the EPM. In conclusion, RSV treatment in diabetic mice provoked anxiolytic-like effects in both tests (OFT and EPM), and these effects were observed in a short time window (2 weeks). It is suggested that RSV may help diabetic animals to adapt to new stressing and anxiety situations and thus to improve their welfare.

Key words: diabetes, flavonoid, mouse, resveratrol, stress

Introduction

Diabetes mellitus is a complex endocrine-metabolic and multifactorial disorder [59]. Diabetes provokes alterations in the central nervous system (CNS) and adversely affects behavior, causing depression [4], cognitive dysfunction [3, 63], increasing in fear-related behaviors [50], and stress response, as shown by an increase in hypothalamo-pituitary-adrenal axis activity [24, 67]. Studies performed in rodents with streptozotocin-induced diabetes type-1 have shown an increase in anxiety-like behaviors in Open Field Tests (OFT) and

the Elevated Plus Maze (EPM) [55, 62].

In the last decade, it has been reported that resveratrol (RSV) has important implications for diabetes treatment [45, 46]. RSV is a natural flavonoid found in the peel of red grapes, red wine, and peanuts and is well known for its antioxidant, cardioprotective, and anticarcinogenic properties [9, 27, 33]. In mice, RSV improves mitochondrial function, energy balance, and life span under caloric restriction by stimulating the Sirt1-mediated PGC-1 α deacetylation [8, 38, 48, 64]. Brain PGC-1 α deficiency in mice leads to behavioral abnormalities, including profound hyperactivity and neurodegeneration

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disease [51, 53]. Some works in animal models have shown that RSV provides neuroprotection and learning rescue in CNS degeneration and motor disorders [20, 31, 44]. Xu *et al.* [81] showed that RSV exerts antidepressant-like effects in mice. These effects might be related to an increase in certain neurotransmitters (for example, serotonin) in different brain areas, such as the hippocampus. It is known that the hippocampus is a brain area associated with anxiety behavior [7, 82]. Also, the serotonergic system is associated with anxiety behavior [11]. In addition, Zhang *et al.* [83] found that certain natural flavonoids exert anxiolytic effects in rats subjected to posttraumatic stress. Moreover, Schmatz *et al.* [68] suggested that RSV can improve learning and memory in diabetic rats.

The aim of the present work was to determine the effect of RSV on the behavioral performance in anxiety tests of streptozotocin-induced diabetic mice after 2 and 4 weeks of treatment.

Methods

Animals

Twenty-four male mice (CD1) were used in this study. All mice were housed in a controlled environment with a 12 h light and 12 h dark cycle and average temperature of $22 \pm 2^\circ\text{C}$ with free access to chow and water. The mice were weaned at 21 days of age and arranged in groups of four to six animals per cage. The experimental procedures were approved by the local Ethics Committee (CEUA IIBCE, Uruguay), in accordance with national legislation.

Induction of diabetes and determination of body weight and glycemia

Type 1 diabetes was induced in 40-day-old mice by administration of a single intraperitoneal streptozotocin (STZ) injection (150 mg/kg body weight, dissolved in 0.1 mmol/l sodium citrate buffer, pH 4.5, Sigma Chemical Co., St. Louis, MO, USA). Body weight and glycemia levels were measured each week following STZ injection. Glycemia determinations were carried out with an Accu-Chek Active system (Roche, Mannheim, Germany), and blood samples were obtained from the tail vein. Mice showing glycemia levels higher than 250 mg/dl one week after STZ injection were considered diabetic.

Resveratrol treatment

Two weeks after STZ injection, diabetes was confirmed in all animals, and the mice were randomly divided into two groups: Diabetic Mice (DM, $n=12$) and Diabetic Mice treated with RSV (RDM, $n=12$). Time zero (week 0) was considered the moment of the start of RSV treatment. RSV (Enzo Clinical Labs, Farmingdale, NY, USA) was freshly prepared in dimethyl sulfoxide (DMSO) with a concentration of 65 mg/ml and administered by intraperitoneal injection (40 mg/kg) every other day for 4 weeks to animals in the RDM group [28, 84]. For a review, see Athar *et al.* [5] and Saiko *et al.* [66]. In parallel, DMSO solution was administered to DM mice as a control.

Behavioral experiments

Mice were brought to the experimental room in their home cages two hours before the behavioral experiments to allow them to acclimate to the test environment. The experimental room was kept at a controlled temperature ($22 \pm 2^\circ\text{C}$). DM and RDM mice were tested in the OFT and EPM in the 2nd and 4th weeks of RSV treatment. This experimental design was similar to the one carried out by Doron *et al.* [29] and Kajiyama *et al.* [40]. The EPM test was performed two days after the OFT in the corresponding week, similar to as reported by Uriarte *et al.* [78]. Both behavioral tests were scored and recorded using a Sony HD camera for further analysis.

Open field test

The OFT apparatus consists of a square Plexiglas cage ($35 \times 35 \times 40$ cm) with walls to minimize outside light and noise. The animals were individually placed in the center of the OFT apparatus and were left to move freely during a 10 min period. The measured behaviors are described in Table 1. Locomotion data including distance moved (m), duration (s) in peripheral zone (a 5.0 cm region in the outer margin of the box), duration (s) in the center zone (12.0×12.0 cm), duration (s) during which the mice were making movements associated with locomotion or displacement (TMLM), and velocity (cm/s) were scored and recorded automatically over the course of 10 min by a camera connected to a computer equipped with the Ethovision XT 7.0 software (Noldus, Wageningen, The Netherlands). After behavioral monitoring, the floor was cleaned with 70% ethanol solution and left to dry before testing the next animal.

Table 1. List of behaviors observed and their respective descriptions

Behavior	Description
Grooming	Number of times that an animal preened its fur or tail with its mouth or forepaws
Rearings	Number of times that a mouse reared up on its hindlimbs irrespective of whether the animal showed on- or off-wall rearing
Defecations	Number of fecal boli
Urinations	Number of times a mouse urinated

Elevated plus maze test

The EPM device consisted of two open and two closed arms (open arms, 30 × 5 cm; closed arms, 30 × 5 cm, surrounded by 15-cm-high walls). The apparatus was made of wood and elevated 40 cm above the floor. Mice were placed on the central platform (5 × 5 cm) of the maze, facing towards a closed arm and allowed to explore the maze for 5 min. The behavioral parameters scored during the experimental sessions were frequency of grooming, rearing, defecations, urinations, frequency of entries into closed (ECA) and open arms (EOA), and percentage of open arms entries (%EOA) [32].

Statistical analysis

The behavioral parameters without a normal distribution (frequency of grooming, rearing, defecations, urinations, and frequency of ECA, EOA, and %EOA) were compared between DM and RDM mice using the Mann–Whitney U test. The behaviors with a normal distribution, such as the distance moved, duration in peripheral zone, duration in center zone, TMLM, and velocity, were compared between DM and RDM mice using the unpaired Student's *t*-test. Body weight and glycemia were analyzed with an ANOVA for repeated measurements. The model considered the following parameters: group (DM or RDM), time (weeks), and the interaction between group and time as fixed effects and individuals in each group as a random effect. Post hoc comparisons were done with the least significant difference (LSD). The data are expressed as means ± standard error of the mean (SEM). Results were considered significant with an alpha level of 0.05 and were considered to show a tendency between 0.05 and 0.1.

Results

Body weight and glycemia

There was no effect of group ($P=0.91$), time ($P=0.81$), or interaction between group and time ($P=0.15$) on body weight of mice (Fig. 1A). Glycemia increased signifi-

cantly over time after the STZ injection ($P<0.0001$), reaching the peak values at week 0 (two weeks after STZ injection) and remaining at high levels and unchanged up to four weeks (Fig. 1B). There was no effect of group ($P=0.98$) or interaction between group and time ($P=0.78$) on glycemia levels in mice.

Behavioral performance

The effect of RSV treatment on diabetic mice in the OFT is presented in Table 2. After two weeks of treatment, the RDM group showed a higher grooming frequency, lower defecation frequency, and a tendency to have a lower duration in the center zone than the DM group (Table 2). In the 4th week of RSV treatment, only a tendency for a lower TMLM was observed in the RDM group compared with the DM group (Table 2).

The effect of RSV treatment in diabetic mice on grooming, rearing, defecation, and urination behaviors in the EPM is presented in Table 3. The RDM group showed a higher frequency of grooming and lower frequency of rearing than DM group in the 2nd week of treatment (Table 3). The RDM group had a tendency to show a higher frequency of entries in open arms, lower entries in close arms, and higher percentage of entries in open arms than the DM group after 2 weeks of treatment with RSV (Figs. 2A, B, and C, respectively). In the 4th week of RSV treatment, the only significant effect observed was a higher grooming frequency in the RDM group than in the DM group (Table 3).

Discussion

In the present report, we provide experimental evidence from anxiety tests suggesting that RSV treatment changes behavioral profiles in mice with STZ-induced diabetes. As mentioned, diabetes was induced in mice by administration of STZ, which produces selective necrosis of pancreatic beta cells and results in an insulin deficiency state. This is a well-characterized animal model used to induce type I diabetes [2,39, 54, 57, 70,

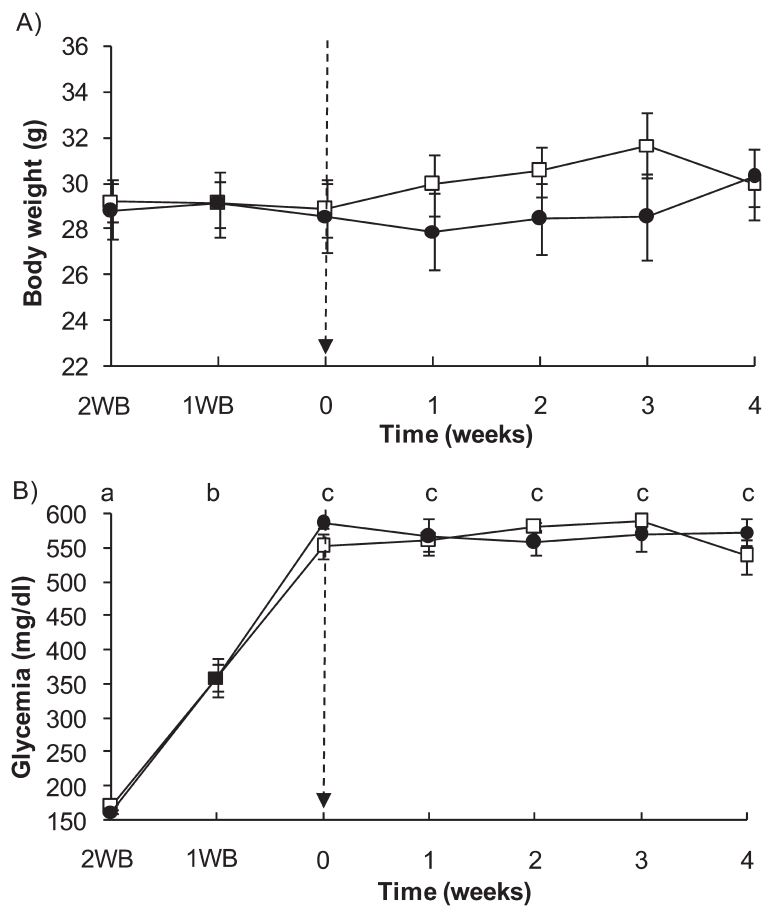


Fig. 1. Body weight (A) and glycemia (B) in diabetic mice with or without RSV treatment (white square and black circle respectively). Two weeks before (2WB) RSV treatment, STZ was injected. The dotted arrow indicates the start of RSV treatment (week 0). Different letters between weeks indicate significant differences at $P < 0.0001$.

74]. In fact, all diabetic animals used in the present report showed high blood glucose levels (above 250 mg/dl, see Fig. 1B) and developed symptoms of severe diabetes characterized by polyuria and polydipsia. By using this animal model to induce type I diabetes, it was observed that RSV treatment produced anxiolytic-like effects in both OFT and EPM. These effects were shown in the OFT by the higher grooming frequency, lower defecation, and lower rearing frequency in the RDM group compared with the DM group and in the EPM test by a significantly higher grooming frequency, a tendency to show a higher frequency of entries in open arms, lower entries in close arms, and higher percentage of entries in open arms in the RDM group than in the DM group. These results agree with the study of Zhang *et al.* [83], who found that certain natural flavonoids exert anxiolytic

effects in rats subjected to posttraumatic stress disorder in 3–15 days. In the present study, it was also observed that the effect on most of the behaviors was recorded mainly in a short time window (2 weeks) of treatment in both tests. After four weeks of resveratrol treatment, there was no effect on most of the behaviors recorded.

As previously mentioned, diabetic type 1 rodents have anxiety-like behaviors augmented in the EPM [1, 62]. This state of anxiety in diabetic animals is associated with changes in the central dopaminergic and serotonergic systems [1, 15, 25, 47, 61, 76]. Xu *et al.* [81] observed that RSV increased dopamine and serotonin levels in several mouse brain areas. Therefore, the anxiolytic effect of RSV in diabetic mice may be explained, partly at least, by its actions on the central dopaminergic

Table 2. Behavioral response of DM and RDM mice in the OFT in the 2nd and 4th weeks after RSV treatment

Behavior	Resveratrol treatment in diabetic mice			
	2nd week		4th week	
	DM	RDM	DM	RDM
Grooming	2.4 ± 0.2 ^a	4.7 ± 0.9 ^b	2.6 ± 0.6	2.7 ± 0.5
Rearing	47.3 ± 5.3	46.5 ± 4.9	41.7 ± 6.5	28.6 ± 5.3
Defecations	3.4 ± 0.4 ^a	1.9 ± 0.3 ^b	3.3 ± 0.7	1.8 ± 0.5
Urinations	0.8 ± 0.1	0.5 ± 0.2	0.8 ± 0.2	0.8 ± 0.2
Distance moved (m)	29.6 ± 2.3	26.3 ± 1.6	25.5 ± 3.1	19.5 ± 2.6
Duration in peripheral zone (s)	518.9 ± 11.1	509.3 ± 19.5	532.4 ± 11.4	537.8 ± 13.2
Duration in center zone (s)	10.4 ± 2.3 [†]	5.5 ± 1.0	5.6 ± 1.2	3.5 ± 0.9
TMLM (s)	379.6 ± 15.9	352.5 ± 16.2	310.0 ± 18.9 [†]	246.6 ± 26.4
Velocity (cm/s)	4.9 ± 0.4	4.4 ± 0.3	4.3 ± 0.5	3.3 ± 0.4

TMLM: time during which the mice were making locomotion movement. Different letters between the DM and RDM groups in the same week indicate significant differences ($P < 0.05$, †: $P = 0.07 - 0.08$).

Table 3. Behavioral response of DM and RDM mice in the EPM in the 2nd and 4th weeks after resveratrol (RSV) treatment

Behavior	Resveratrol treatment in diabetic mice			
	2nd week		4th week	
	DM	RDM	DM	RDM
Grooming	1.4 ± 0.3 ^a	5.3 ± 1.3 ^b	2.3 ± 0.8 ^a	5.2 ± 1.1 ^b
Rearing	24.5 ± 1.5 ^a	11.3 ± 2.4 ^b	15.3 ± 2.3	15.5 ± 2.5
Defecations	0.5 ± 0.2	0.3 ± 0.2	0.3 ± 0.3	0.3 ± 0.2
Urinations	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.0 ± 0.0

Different letters between the DM and RDM groups in the same week indicate significant differences ($P < 0.05$).

and serotonergic systems.

In rodents, an increase in fear response has been associated with an increase in the frequency of defecation in both the OFT and EPM test [10, 21, 35]. According to our results, RSV reduced the defecation frequency in the OFT in the 2nd week of treatment, suggesting that RSV provoked a diminished fear and stress response in diabetic mice. Although the difference was only observed in the OFT, decrease in the frequency of defecation in RDM mice may also be explained by the dopaminergic status [19].

It should be noted, however, that grooming is a behavior that may manifest as body care, self-calming procedure, displacement activity, and comfort in rodents [13, 72, 77]. The greatest grooming increase in the RDM group resulted in a better appearance and body care than in the DM group (data not shown). Even the higher frequency of grooming in the RDM group than in the DM group was maintained until the fourth week of treatment with RSV during the EPM. Since this grooming behavior is maintained over time and is related to body care,

it seems to be one of the most important behavioral changes triggered by RSV in diabetic mice. Several studies in rodents have shown that environmental enrichment increases grooming behavior [16, 17, 60]. In a recent study, it was observed that grooming behavior was linked with an anxiolytic state in rodents [10]. The high grooming activity observed in RDM mice may be a way in which the rodent attempts to escape, to resolve, or to adapt to tension or anxiety situations [36, 41, 56, 65]. It has been reported that the dopaminergic status is implicated in the manifestation of grooming behavior [49] and that this behavior is partly regulated by dopamine D1 receptors [12, 23, 28]. Since dopamine D1 agonists induced an increase in grooming [14, 73] and RSV increased dopamine and serotonin levels in different areas of mice brain [81], it may be possible that RSV increased grooming activity in the RDM group due to a dopaminergic effect. On the other hand, it was found that 5-hydroxytryptamine (5-HT) agonists increase grooming and have an anxiolytic effect in rodents [6, 37, 43]. Therefore, similar effects were reported for the serotonergic

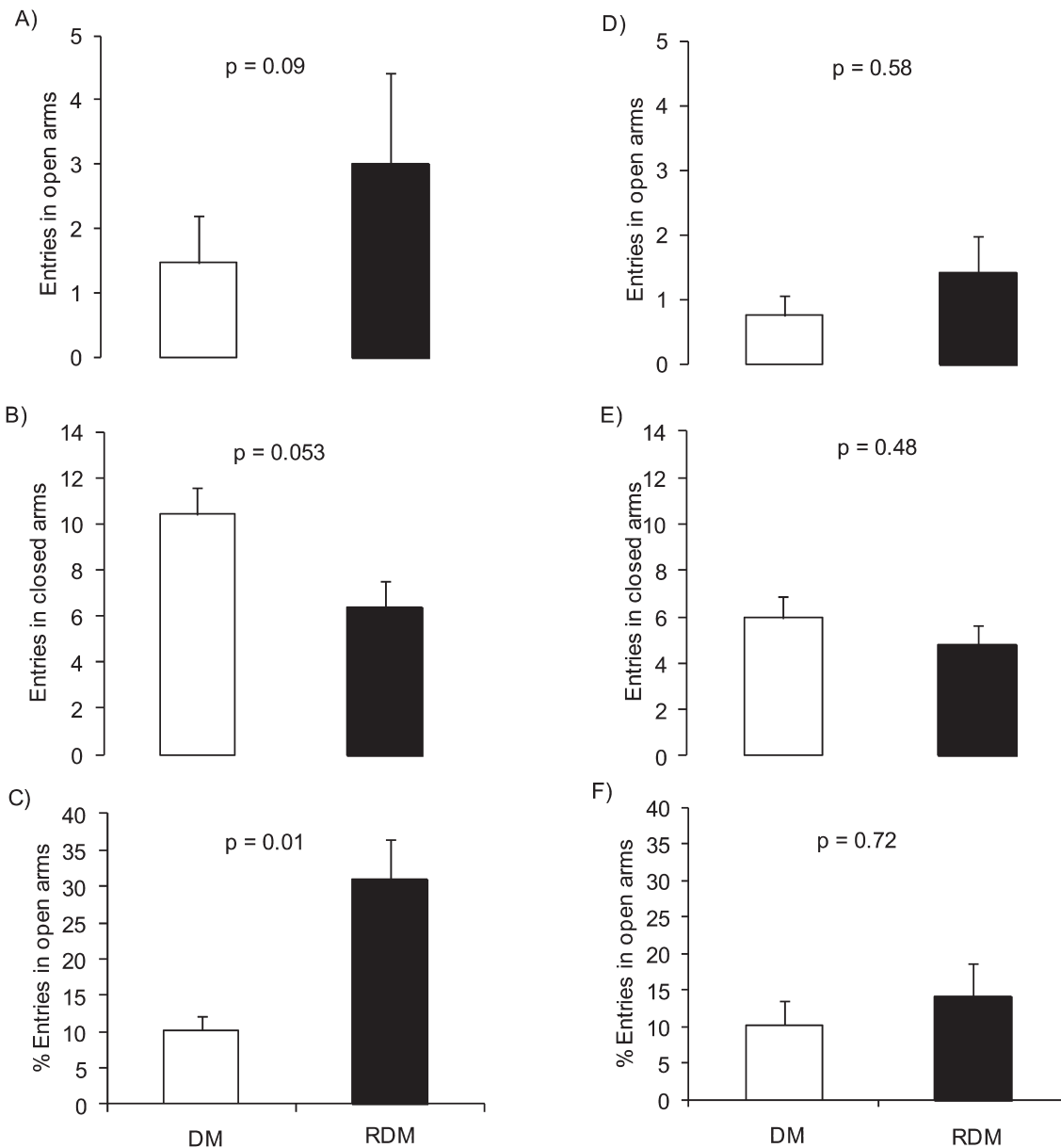


Fig. 2. Frequency of behaviors (mean \pm SEM) recorded in the EPM in the 2nd (A, B, and C) and 4th weeks (D, E, and F) in diabetic mice with or without RSV treatment (RDM and DM respectively).

system. Thus, the increase in grooming frequency caused by resveratrol can also be explained by higher levels of 5-HT in different brain areas, as it has been shown in mice by Xu *et al.* [81]. However, some authors propose that the grooming behavior in rodents partially explains anxiety states [42]. Taking all the OFT and EPM tests results together, we suggest that the higher grooming frequency in diabetic mice is associated with an anxiolytic state caused by RSV.

While anxiogenic drugs may decrease the rearing fre-

quency [22], another study reported that fluoxetine (anxiolytic drug) can decrease the frequency of this behavior [30]. Beyond the different interpretations it is known that rearing is a normal behavior of the exploration performed by rodents when placed in a new environment [52]. Since the DM group tended to remain longer in the closed arms than the RDM group, it is difficult to analyze rearing behavior results. RDM mice performed less rearing, but this may be related to the shorter period of time they were in these arms.

In our study, RSV did not alter body weight or glycemia in diabetic mice. Our results regarding the effect of RSV on body weight in diabetic mice are in agreement with those reported by Schmatz *et al.* [68, 69] in diabetic rats. However, Chen *et al.* [26] observed that diabetic rats treated with RSV had an increased body weight when compared with diabetic rats without treatment. Nevertheless, it is difficult to reach conclusions because these experiments are not totally comparable, as different dosage methods were used: intraperitoneal [68, 69] and oral administration [26]. Our glycemia results in diabetic mice are also consistent with those obtained by Schmatz *et al.* [68, 69] in rats, and both studies administered RSV intraperitoneally. However, using the same RSV intraperitoneal administration method, Silan [71] observed a blood glucose lowering effect in diabetic rats. This effect was also observed by Thirunavukkarasu *et al.* [75] and Penumathsa *et al.* [58] in studies in which resveratrol was given orally to STZ-induced diabetic rats.

The anxiolytic-like effect of RSV in diabetic mice was mainly observed in a short time window (2 weeks). The absence of an RSV effect on anxiety behavior in long-term diabetic animals may be explained in part by chronic hyperglycemia. It is well known that long-term diabetic animals develop chronic hyperglycemia, which may provoke great functional brain changes [80], and these harmful effects may be stronger than the positive RSV effects.

It is important to point out that the animals used in the present report had high levels of glycemia and were not treated with insulin. For this reason, future studies are needed to evaluate if RSV affects anxiety behavior in streptozotocin-induced diabetic mice with insulin treatment. In addition, it will be interesting to know if systems apart from the dopaminergic and serotonergic ones have an effect on the diabetic animal's behavior when RSV is administered.

Other important aspects to evaluate could be the doses and alternative routes of RSV administration. In this sense, Vissienon *et al.* [79] worked with others flavonoids (such as kaempferol and quercetin) in mice and found that the anxiolytic effect was observed only after oral administration, while this effect was not observed after intraperitoneal administration. The authors suggested that these flavonoids can be transformed to active metabolites by intestinal microbiota. In the same work, the authors also observed the effect of doses of

the respective flavonoids (kaempferol and quercetin) on anxiety behavior in mice. Both flavonoids showed an anxiolytic effect at the concentrations of 0.5 and 1.0 mg/kg administered by the oral route, but no anxiolytic effect was observed at lower (0.1 mg/kg) or higher doses (2.0 mg/kg). For the specific case of RSV, it was reported that it provokes changes at the CNS level under oral or intraperitoneal administration [34, 68, 81]. Gacar *et al.* [34] administered different intraperitoneal doses of RSV in rats and observed that only the higher doses (50 mg/kg) caused the spatial memory in the Morris water maze test to improve, while no effects were observed with lower doses (12.5 and 25 mg/kg). Schmatz *et al.* [68] suggest that RSV (intraperitoneal administration at 10 and 20 mg/kg) can improve learning and memory in diabetic rats. In addition, Xu *et al.* [81] observed that RSV (oral administration in mice) increased dopamine and serotonin levels in several mouse brain areas in a dose-dependent manner. However, it will be necessary to perform more studies in diabetic animals using several doses of RSV by different routes of administration to determine its effects on anxiety behavior.

The welfare of an individual was defined by Broom [18] as "its state as regards its attempts to cope with its environment." Since in our study the RSV had an anxiolytic effect in diabetic mice, it is suggested that RSV may help diabetic animals to adapt to new situations of stress and anxiety and thus to improve their welfare.

In conclusion, RSV provoked anxiolytic-like effects in streptozotocin-induced diabetic mice, and these effects were observed in a short time window (2 weeks) of treatment. It is suggested that RSV may help diabetic animals to adapt to new situations of stress and anxiety and thus to improve their welfare.

Conflicts of interest

There are no conflicts of interest.

Acknowledgments

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