Insulin supplemented with phenolic fraction concentrates displays anxiolytic and antidepressant-like properties with reductions of oxidative brain damage in chronically stressed diabetic rats

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A B S T R A C T

Introduction: The current study aimed to investigate if insulin supplemented with phenolic fraction concentrates (PFC) improves chronic hyperglycemia-related behavioral changes by mitigating oxidative stress in diabetic rats exposed to chronic mild stress (CMS).
Methods: Experimental type 1 diabetes mellitus (T1DM) was established by a single intraperitoneal injection of streptozotocin (STZ, 65 mg/kg). After diabetes confirmation, rats were treated with insulin supplemented with PFC and exposed to two unpredictable mild stressors per day for 12 weeks. Body weight changes, fasting blood glucose (FBG), and corticosterone levels were evaluated. The behavioral tests were performed to evaluate anhedonia, anxiety, and depressive-like behaviors. Twenty-four hours after behavioral tests, all rats were anesthetized, and the blood was collected for the analysis of lipid, hepatic, and renal parameters. Finally, the brain areas (striatum, hippocampus, and prefrontal cortex), pancreas, and adrenal glands were dissected for the analysis of oxidative stress markers.
Results: The results of this study revealed that treatment with insulin supplemented with PFC for 12 weeks significantly enhanced antioxidant defenses (catalase [CAT] and superoxide dismutase [SOD]) and reduced oxidative stress damage (nitric oxide and malondialdehyde [MDA]), especially in brain regions (prefrontal cortex, hippocampus, and striatum) in stressed diabetic rats ($P < 0.001$). This combination also ameliorated the corticosterone level ($P < 0.001$) as well as glucose homeostasis ($P < 0.001$) and lipid parameters ($P < 0.001$), which are markedly altered in T1D associated with stress.
Conclusion: The associated treatment possesses important anxiolytic and antidepressant-like effects in this rat model, which might be mainly mediated by its capacity to protect brain cells against reactive oxygen species (ROS) triggered by T1DM and/or chronic stress.

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Introduction

The concurrence of type 1 diabetes mellitus (T1DM) and psychiatric disorders constitutes to be a serious health problem worldwide. Chronic hyperglycemia is considered a significant risk factor for the evolution of anxiety and depression, especially among individuals with T1DM (1). Till now, an overlap in the physiopathology of psychiatric disorders and T1DM has been reported in neuroimaging studies (2). These findings are supported by investigations in animal models that allow better understanding and elucidation of the physiopathological consequences induced by chronic hyperglycemia (3).

Previous investigations have clearly revealed that T1DM can lead to various complications in different areas of the central and peripheral nervous systems (4). Oxidative stress may result from decreased antioxidant defense enzymes, overproduction of reactive oxygen species (ROS), or a failure to regulate existing oxidative damage (5). In T1DM-induced chronic hyperglycemia, a crucial feature of prolonged exposure to high, blood sugar levels are the overabundance of ROS in organs within both the peripheral and central nervous systems (6). These ROS can then induce cell injury through lipid peroxidation generation, DNA modification, and enzyme inactivation, specifically targeting the more vulnerable brain tissues (7). Consequently, ROS accumulation may play a significant role in the pathogenesis of several psychiatric disorders (8). The behavioral alterations induced by chronic hyperglycemia might be ascribed to numerous other factors, including reduced monoamine neurotransmitters concentrations in many brain regions and synaptic plasticity deteriorations (4,9).

Stress is a crucial contributor to physiopathological situations in humans. Individuals with chronic diseases such as type 1 diabetes face several episodes of stress, and thus it is important to determine the differential impacts that general life stress might have when compared to disease-related specific stress in order to develop more suitable interventions (10). The rats with normoglycemia exposed to chronic mild stress (CMS) had elevated oxidative stress products and reduced antioxidant enzyme activities in various brain regions, including the hippocampus (11). Changes in hormones that occur during chronic stress conditions can also significantly affect glucose homeostasis, especially in patients with diabetes (12). Additionally, the prolonged secretion of corticosterone in the hyperglycemic state results in increased exposure of nerve cells to corticosterone, which is known to result in dendritic atrophy, decreased synaptic plasticity, and reduced neurogenesis, leading to neuronal damage. Each of these can promote neuropsychiatric disorders (13,14). As can be expected, therapy with antioxidant compounds that alter ROS mechanisms can serve to decrease the complications of diabetes development by reducing the effects of oxidative stress (15).

In particular, one such natural antioxidant is found in the date (*Phoenix dactylifera*) seed. The date seeds represent an important part of the fruit. It is mainly rich in phenolic compounds, and several studies have reported the powerful antioxidant activity that date seeds exhibit (16). The antioxidant effect is suggested to be, in large part, due to the phenolic fractions contained within the seeds (16). Phenolic fraction concentrates (PFC) extracted from date seeds has also been revealed to have many benefits against diabetes and its complications, including the inhibition of digestive enzymes (16). In addition, date seeds extract may significantly enhance the activity of endogenous antioxidants such as glutathione peroxidase, superoxide dismutase (SOD), and catalase (CAT) (17), and consequently, attenuate ROS products in various organs (18). To the best of our knowledge, there are no studies that have assessed the behavioral alterations that result from the oxidative environment induced by T1D and stress or how phenolic fractions concentrates (PFC) of the date seed may have neuroprotective effects against oxidative damage in type 1 diabetics under stress. Therefore, the main objective of the current study was to investigate if insulin supplemented with PFC improves chronic hyperglycemia-related behavioral changes by mitigating oxidative stress in diabetic rats exposed to CMS.

Material and Methods

Preparation of phenolic fraction concentrate

Date seeds phenolic fraction concentrate extraction was performed as previously described in our published study (16). In brief, date seeds were dried in the oven (50°C for 48 hours) and finely ground until the powder was obtained (<1.5 mm). The powder was macerated with acetone (50%) and acetic acid (2%) with a sample/solvent ratio (1:10). The macerate was then subjected to extraction for one hour at 45°C. The obtained extract was centrifuged and evaporated to dryness at 60°C. To purify the phenolic fractions from the crude extract obtained, N-butanol was used (funnel separation was used). Then, the obtained phenolic fraction was evaporated at 60°C, and finally dried in an oven at 60°C and ground into powder.

Animals

Experiments were performed on Wistar rats (300–320 g), taken from the central animal facility of the Faculty of Sciences of Kenitra, Morocco. They were housed under the standard light/dark: 12/12 cycle, at constant temperature of 23°C with free access to food and water. Experiments were conducted in accordance with the principles of the "Guide for the Care and Use of Laboratory Animals" published by the National Institute of Health.

Experimental diabetes induction

We used the streptozotocin (STZ)-induced diabetic animal

model to produce T1D in rats (19). Overnight fasting rats received an intraperitoneal (i.p.) injection of 65 mg/kg STZ (Sigma. St Louis. MO. USA). After the infusion with STZ, animals were given aqueous glucose (5%) for only 12 hours. To confirm hyperglycemia, we measured glucose levels in blood samples 3 days after the injection. Only rats that had their blood glucose level greater than or equivalent to 250 mg/dL were considered diabetic and chosen for this investigation.

Chronic mild stress procedure

The CMS protocol was preceded as described previously (20), with slight modifications. Briefly, each day from 8:00, STZ-diabetic rats were exposed to 2 unpredictable mild stressors for 12 weeks (6 days/week). The mild stressors included cold stress (4°C, 1 hour), water deprivation (24 hours), tilted cage (45°, 24 hours), forced swimming (18°C, 5 minutes), crowding (5 animals within one cage, 24 hours), tail clamp (1 minute), inversion of light/dark cycle (24 hours), restraint (4 hours), overnight illumination (12 hours) and wet bedding (24 hours).

Experimental design

After the confirmation of hyperglycemia, rats (n=48) were divided into four experimental groups: STZ-diabetic group (Diabetic control: 1 mL 0.9% NaCl /200 g/d); STZ-diabetic group: exposed to CMS (STZ+CMS: 1 mL 0.9% NaCl/200 g/d); STZ-diabetic group: exposed to CMS and treated with insulin (6 UI) (STZ+CMS+INS, 1 mL 0.9% NaCl /200 g/d); STZ-diabetic group: exposed to CMS and treated with insulin (6 UI) supplemented with phenolic fractions concentrate (50 mg/kg) (STZ+CMS+INS+PFC). Another group (n=12) was kept as normal control (NC: 1 mL 0.9% NaCl/200 g/d). The dose of insulin used in this study was based on a previous study (21). The PFC dose used (50 mg/kg) was based on our previous study reporting the in vitro antioxidant and anti-hyperglycemic activities of PFC and acute and subacute oral toxicity studies (16).

During the experiment, body weight changes and fasting blood glucose (FBG) were measured during three periods, viz. T0 (hyperglycemia confirmation day), T1 (after 6 weeks of treatment), and T2 (after 12 weeks of treatment). Plasma corticosterone levels were also evaluated in two periods, T1 and T2. Blood samples were taken from the caudal vein for FBG and corticosterone levels evaluation. Moreover, at T1 and T2, the behavioral tests were performed to evaluate anhedonia, anxiety, and depressive-like behaviors. Twenty-four hours after behavioral tests, all rats were anesthetized with chloral hydrate before sacrifice, and the blood was collected for lipid, hepatic, and renal parameters analysis. Finally, the brain regions (striatum, hippocampus, and prefrontal cortex) and peripheral organs, viz. pancreas and adrenal gland, were dissected out for oxidative stress markers analysis.

Behavioral tests

Sucrose preference test

The sucrose preference test (SPT) was proceeded as described previously (22). Rats were housed in individual cages with two bottles of sucrose solution (1%, w/v) in each cage for 24 hours. Then, one of the bottles was replaced with water for another 24 hours for the adaptation of rats to the presence of the two bottles. During the test day, rats were deprived of water and food for 1 hour, and the test was performed at 9:00 AM. Rats were given free access to the two bottles of the same shape, one containing 100 mL of sucrose solution 1% and the other 100 mL of water. After 4 hours, we measured the volume (mL) of both consumed sucrose solution and water.

Elevated plus maze test

The elevated plus maze (EPM) test was performed as described previously (22). Animals were tested randomly; each rat was placed in the center of the apparatus facing one of the open arms and was recorded for 5 minutes. The parameters calculated for this test were the percentage of open-arm entries and the percentage of time spent in the open arms.

Open field test

To investigate the anxiety-like behavior in rats, the open field test (OFT) was performed as described previously (23). Briefly, during the test day, each rat was placed on the central platform and allowed to explore the apparatus freely for 10 minutes. We measured the number of the central platform's visits as well as the time spent in it, which is a measure of anxiety.

Light-dark box test

The light-dark box test (LDBT) is a two compartments apparatus: one is dark and the other is highly illuminated, connected through a tunnel. The LDBT is used to assess the rats' reactions in a highly illuminated environment. Anxious rats spend less time on the illuminated side. Animals are individually placed on the dark side facing the door. We measured the time spent in the white compartment for 6 minutes (23).

Forced swimming test (FST)

Rats were placed individually in a tank (30 cm × 40 cm height) filled with 25 cm³ of water at 24 ± 1°C temperature to swim for 15 minutes for a pre-test session. Then, 24 hours later, the animals were forced to swim for 5 minutes. During this test session, we measured the total time of immobility (24).

Biochemical assays

Plasma corticosterone assay

Plasma corticosterone levels were evaluated using rats ELISA kits as described in the manufacturer's instructions. We measured the absorbance at 450 nm using the

microplate reader, and the plasmatic corticosterone value was expressed in ng/mL.

Lipid, hepatic, and renal parameters analysis

Twenty-four hours after the behavioral tests, all rats were anesthetized with chloral hydrate, and blood samples were collected and centrifuged for 10 minutes at 4°C at 4000 rpm speed. The plasma was then collected in tubes for spectrophotometric analysis of lipids, including total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and total triglycerides using enzymatic diagnostic kits (DiaSys, System diagnostic GmbH, Germany) as described in the manufacturer's protocols. Then, we calculated the very low-density lipoprotein cholesterol (VLDL-C) using the formula: VLDL-C = (total triglycerides/5). We also calculated the atherogenic index of plasma (AIP) and cardiovascular risk indices as follow: AIP = Log10 (TG/HDL-C), cardiovascular risk index 1 = TC/HDL-C and cardiovascular risk index 2 = LDL-C/HDL-C. Furthermore, we evaluated the levels of creatinine, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using specific diagnostic kits according to the manufacturer's instructions (DiaSys, GmbH, Germany).

Preparation of homogenates

After sacrificing the rat, the prefrontal cortex, striatum, and hippocampus of each hemisphere were collected and separately dissected. The Dounce homogenizer was used to homogenize tissues in an ice-cold lysis buffer (RIPA lysate solution + 1 mM PMSF), and the homogenates were centrifuged for 15 minutes (14 000 g), then stored at −80°C (23). The pancreas and adrenal gland tissues were prepared following the protocol described by Erejuwa et al (25). Briefly, ten percent (w/v) tissue homogenate was prepared in Tris-HCl (0.1M, pH 7.4) using an ice-chilled homogenizer vessel. The suspended mixture was then centrifuged at 1000 × g for 10 minutes at 4°C in a refrigerated centrifuge.

Assessment of oxidative stress markers
Determination of superoxide dismutase activity

In this study, SOD activity was measured in brain homogenates as described by Beauchamp and Fridovich (26).

The Cayman assay kit was used to measure SOD activity in the pancreas and adrenal gland tissues following the manufacturer's instructions. Tetrazolium salt is used in this kit to detect superoxide radicals generated by xanthine oxidase and hypoxanthine (25). SOD content was measured and expressed in IU/mg of protein, and 1 IU of SOD activity was interpreted as the amount of enzyme needed to inhibit the reduction of NBT by 50% (26).

Determination of catalase activity

CAT activity was measured in the brain homogenates as described previously (27). CAT activity was measured in the pancreatic and adrenal gland homogenates as described by Erejuwa et al (25). One IU of CAT was interpreted as the amount of enzyme needed to catalyze the decomposition of 1 μmol of hydrogen peroxide per minute.

Lipid peroxidation assay

Thiobarbituric-acid-reacting substances (TBARS) level in cells was measured to analyze the formation of lipid peroxides in brain homogenates (28). The 2 mL mixture added to samples consisted of 1 mL of trichloroacetic acid 10% and 1 mL of thiobarbituric acid 0.67%; the whole was heated for 15 minutes in a water bath (90°C). Then, butanol (2:1 v/v) was added to the solution. After centrifugation for 5 minutes at 8000 g, the TBARS level was analyzed by measuring the absorbance at 535 nm (29). Likewise, TBARS measurement was carried out for pancreatic and adrenal gland tissues by mixing the tissue homogenates with 1 mL of trichloroacetic acid 10% and then heating the solution for 60 minutes in the water bath (30). The concentration of the lipid peroxidation was expressed as nmol/mg of protein.

Nitrite/nitrate assay

The concentration of nitrite in the rat tissue homogenates was determined using the diazotization method based on the Griess reaction, which is an indirect technique to measure nitrite oxide (NO) production (31). Nitrite levels were expressed in μmol/g protein. We used the linear regression analysis to calculate nitrite concentrations in the tissue homogenates, using the standard calibration curves of sodium nitrite.

Protein assay

The protein content was measured with bovine serum albumin (0 to 15 mg/mL), as described by Bradford (32), following the manufacturer's protocols.

Statistical analysis

The findings were mentioned as the mean values ± standard deviation (SD) and subjected to statistical analysis using SPSS software. These results were analyzed by one-way ANOVA test, followed by a post hoc test (Tukey's test) for Multiple-group comparisons. The difference was considered significant for values less than 0.05.

Results
Fasting blood glucose and body weight changes

FBG and body weight changes for each group are presented in Figure 1. At baseline (STZ i.p day), there were no significant differences in FBG levels (Figure 1A) and body weights (Figure 1B) between the groups. After 72 hours (T_0) of STZ injection, all rats injected with STZ showed a statistically significant increase in FBG levels associated with a significant decrease in body weight
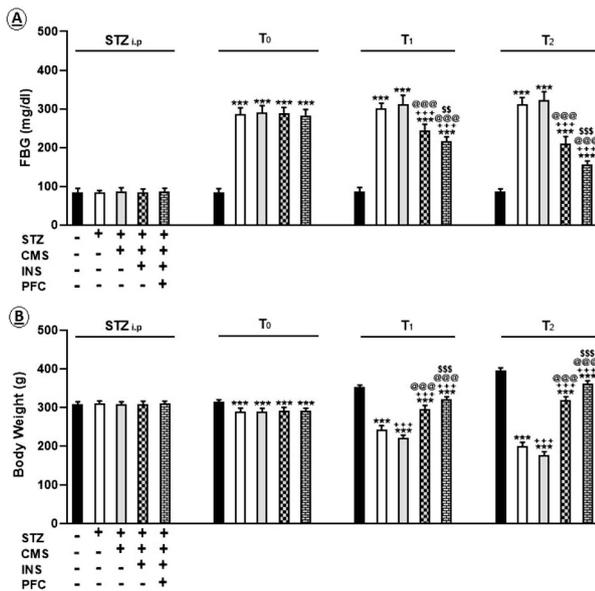
Figure 1. Effects of insulin (INS) supplemented with phenolic fraction concentrates (PFC) on fasting blood glucose (FBG) (A) and body weight (B) changes in STZ-diabetic rats exposed to chronic mild stress (CMS) during three periods, viz. T0 (hyperglycemia confirmation day), T1 (after 6 weeks of treatment), and T2 (after 12 weeks of treatment). Results are expressed as Mean ± SD (n=12). *** $P < 0.001$ vs. normal control. +++ $P < 0.001$ vs. untreated STZ-diabetic group. @@@ $P < 0.001$ vs. untreated STZ-diabetic group exposed to CMS. $$$ $P < 0.001$ vs. STZ-diabetic group exposed to CMS and treated with insulin.

($P < 0.001$). At T_1 (after 6 weeks) and T_2 (after 12 weeks), the treated groups, had significantly lower FBG levels associated with a remarkable increase in body weight than the untreated STZ+CMS group ($P < 0.001$). Moreover, a significant increase in body weight was observed in the same group compared to the untreated STZ+CMS ($P < 0.001$). During the 12 weeks of the experiment, FBG and body weight significantly improved gradually in the stressed diabetic group treated with insulin supplemented with PFC compared to the stressed diabetic group with insulin treatment ($P < 0.001$).

Corticosterone levels evaluation

Corticosterone levels at T1 and T2 for each group are presented in Figure 2. The STZ-diabetic group exhibited a significant increase in plasma corticosterone after 6 and 12 weeks of STZ injection compared to the NC group ($P < 0.001$). After 6 weeks as well as 12 weeks of the CMS exposure, the group injected with STZ and exposed to CMS experienced a remarkable significant increase in corticosterone levels compared to the untreated STZ group ($P < 0.001$). This effect was remarkably prevented by INS treatment when compared to the untreated STZ+CMS group ($P < 0.001$). STZ+CMS+INS+PFC group for 12 weeks showed a notable decrease in corticosterone levels ($P < 0.001$) compared to untreated STZ+CMS and STZ+CMS+INS+PFC groups.

Sucrose preference test

In order to evaluate the effect of INS supplemented with PFC as a treatment for STZ-diabetic rats exposed to CMS on anhedonia, the SPT was used, and the findings are summarized in Figure 3. The SPT results showed a significant difference in the percent of sucrose preference between the STZ-diabetic groups exposed or not exposed to CMS and the normal control group ($P < 0.05$). At 6 weeks, the findings did not reveal any difference between the untreated STZ group and the untreated STZ+CMS group ($P > 0.05$). The STZ+CMS+INS group and the
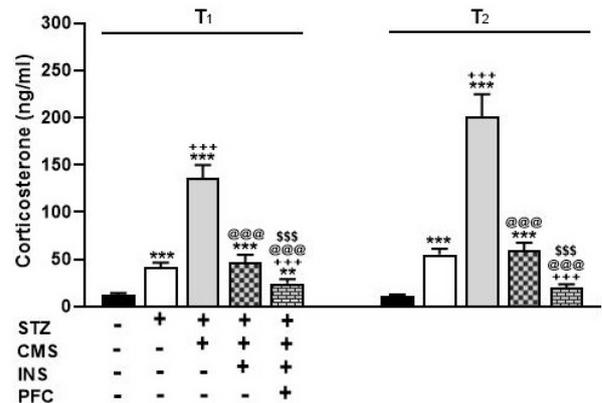

Figure 2. Effects of insulin (INS) supplemented with phenolic fraction concentrates (PFC) on plasma corticosterone levels in STZ-diabetic rats exposed to chronic mild stress (CMS) during two periods, viz. T1 (after 6 weeks of treatment) and T2 (after 12 weeks of treatment). Results are expressed as mean ± SD (N=10-12). ** $P < 0.01$, *** $P < 0.001$ vs. normal control. +++ $P < 0.001$ vs. untreated STZ-diabetic group. @@@ $P < 0.001$ vs. untreated STZ-diabetic group exposed to CMS. $$$ $P < 0.001$ vs. STZ-diabetic group exposed to CMS and treated with insulin.
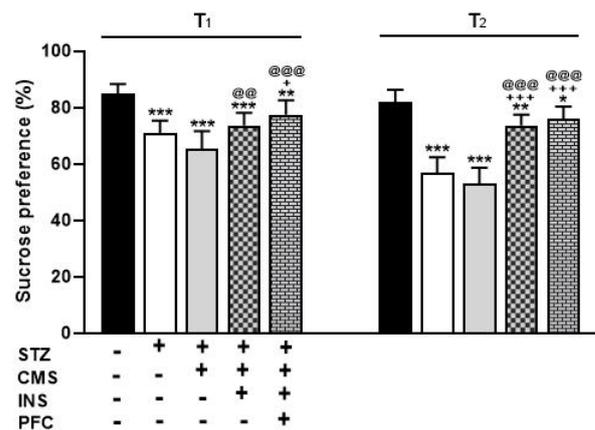

Figure 3. Effects of insulin (INS) supplemented with phenolic fraction concentrates (PFC) on anhedonia behavior in STZ-diabetic rats exposed to chronic mild stress (CMS) during two periods, viz. T1 (after 6 weeks of treatment) and T2 (after 12 weeks of treatment). Results are expressed as mean ± SD (N=10-12). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. normal control. + $P < 0.05$, +++ $P < 0.001$ vs. untreated STZ-diabetic group. @@ $P < 0.01$, @@@ $P < 0.001$ vs. untreated STZ-diabetic group exposed to CMS.

STZ+CMS+INS+PFC groups had a higher percent sucrose preference than the untreated STZ+CMS group ($P<0.01$, $P<0.001$, respectively). Moreover, treatment with insulin only or supplemented with PFC for 12 weeks prevented remarkably the anhedonia symptom when compared to the untreated STZ+CMS group, with $P<0.001$ for both groups.

Anxiety and depression-like behaviors

As depicted in Figure 4, the anxiety-related behaviors analysis of STZ and STZ+CMS groups based on the EPM test revealed a significant decrease in the percentage of open arms entries (Figure 4A) and the percentage of time spent in these arms (Figure 4B) compared to the normal control group ($P<0.001$). The results did not show any significant difference between the STZ and STZ+CMS

groups. Similarly, after 6 weeks (T1) as well as 12 weeks (T2) of treatments, the OF test parameters of STZ and STZ+CMS groups showed a significant decrease in the percentage of time spent in central squares (Figure 4C) and the number of returns to the central squares (Figure 4D) compared to normal control group ($P<0.001$). Insulin treatment significantly increased the evaluated EPM and OF parameters. However, PFC-supplementation resulted in a significant decrease in anxiety-related behaviors in both assays when compared to insulin treatment only ($P<0.01$). Finally, as indicated in Figure 4E, after 6 weeks of the experiment (T1), the anxiety-related behavior analysis of STZ, STZ+CMS, and STZ+CMS+INS groups based on the BOX test revealed a significant decrease in the time spent on the light side compared to NC ($P<0.05$, $P<0.001$, $P<0.01$, respectively). In addition, the results
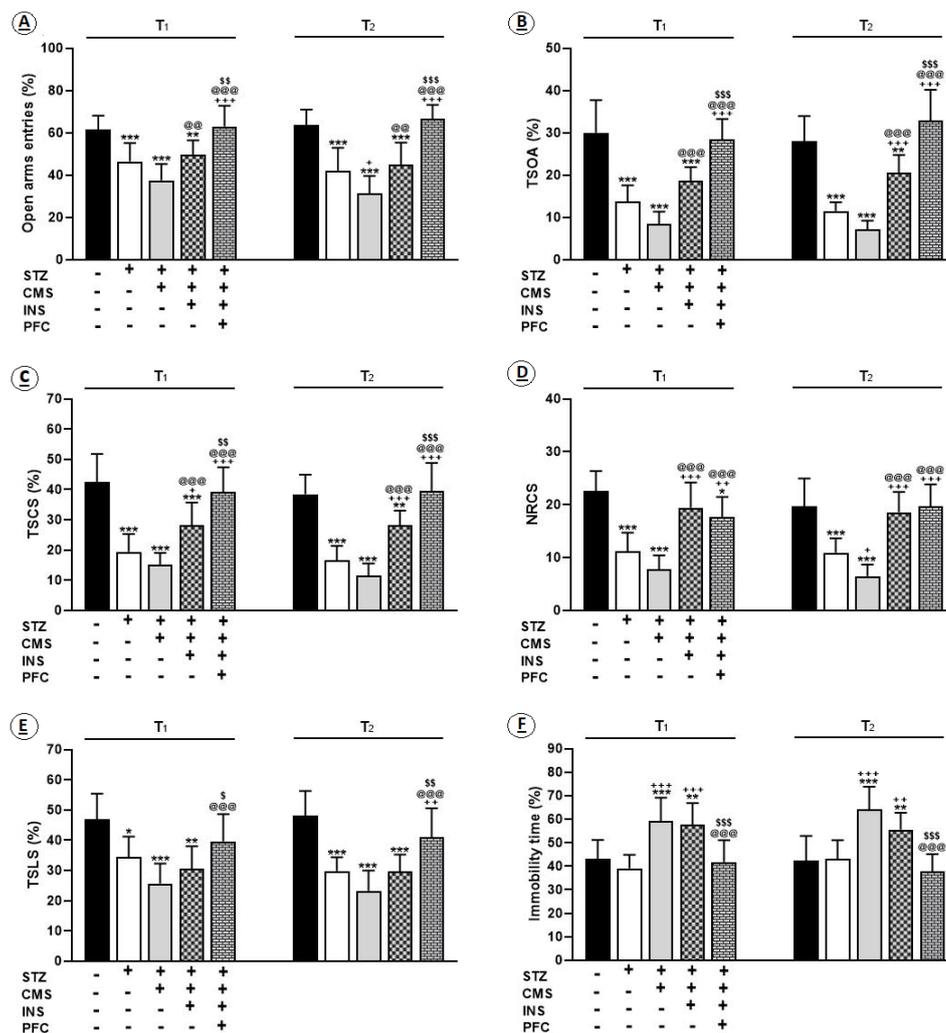

Figure 4. Effects of insulin (INS) supplemented with phenolic fraction concentrates (PFC) on anxiety and depression-like behaviors in STZ-diabetic rats exposed to chronic mild stress (CMS) during two periods, viz. T_1 (after 6 weeks of treatment) and T_2 (after 12 weeks of treatment). (A) Open arms entries in EPM test; (B) Time spent in open arms in EPM test (TSOA, %); (C) Time spent in central squares in OF test; (D) Number of returns to the central squares in OF test (NRCS); (E) Time spent in the light side in light-dark BOX test (TSLS); (F) The percentage of immobility time in FST test. Results are expressed as Mean ± SD (N=10-12). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. normal control. + $P<0.05$, ++ $P<0.01$, +++ $P<0.001$ vs. untreated STZ-diabetic group. @ $P<0.05$, @@ $P<0.01$, @@@ $P<0.001$ vs. untreated STZ-diabetic group exposed to CMS. $$ $P<0.01$, $$$ $P<0.001$ vs. STZ-diabetic group exposed to CMS and treated with insulin.

did not show any significant difference between the STZ, STZ+CMS, and STZ+CMS+INS groups (P>0.05). Likewise, there was a significant decrease in the time spent in light side at T2 for the STZ, STZ+CMS, and STZ+CMS+INS groups compared to the normal control group (P<0.001). However, STZ+CMS+INS+PFC rats showed a significant increase in the time spent on the light side in both points T1 (P<0.05), T2 (P<0.01) when compared to STZ+CMS+INS rats.

The FS test findings are summarized in Figure 4F. At weeks 6 and 12 of the experiment, the results of the FS test revealed that diabetes induced by STZ was not accompanied by depressive-like behaviors in the STZ group compared to the NC group (P>0.05). However, CMS, during 6 and 12 weeks, significantly increased the percentage of immobility time in STZ+CMS and STZ+CMS+INS groups compared to the normal control group (P<0.001 and P<0.01, respectively). PFC-supplementation resulted in a significant decrease in depressive-related behaviors in both time points compared to insulin treatment only (P<0.001).

Biochemical analysis and cardiovascular risk indices evaluation

In order to evaluate the potency and efficacy of the above treatments on different physiological functions in STZ-diabetic rats exposed to CMS, the biochemical parameters (lipid, renal, and hepatic parameters) and cardiovascular risk indices were analyzed (Table 1). After 12 weeks of STZ injection, total cholesterol, triglycerides, LDL-C, VLDL-C, AIP, CVRI-1, CVRI-2, AST, ALT, and creatinine

levels were significantly higher in the untreated STZ- and the untreated STZ+CMS groups compared to the NC group (P<0.001). While, the untreated STZ+CMS group exhibited a notable increase in triglycerides, VLDL-C, ALT, and creatinine when compared with the STZ- group (P<0.05). Therefore, STZ+CMS groups treated with INS only or supplemented with PFC for 12 weeks showed a remarkable decrease in all lipid parameter levels (except a significant increase in HDL-C), as well as renal and hepatic levels compared to the untreated STZ+CMS group.

Oxidative stress markers analysis

The levels of oxidative stress markers and the activities of antioxidant enzymes in peripheral organs (pancreas, adrenal glands) and brain regions (hippocampus, prefrontal cortex, and striatum) of normal and STZ-diabetic rats exposed or/not exposed to CMS were evaluated, and the findings are presented in Figures 5, 6, 7, and 8. At the end of the treatment period, the results revealed a significant increase in the concentrations of malondialdehyde (MDA) and NO in adrenal glands (Figures 7B, 8B), pancreas (Figures 7C, 8C), and brain regions (Figures 7A, 8A) in the untreated STZ-diabetic groups exposed or not exposed to CMS compared with the normal control group (P<0.001). Significant lower activities of CAT (Figures 5A, 5B, 5C) and SOD (Figures 6A, 6B, and 6C) in all the tissues studied were observed in the same groups compared to the normal group (P<0.001). Furthermore, remarkably higher levels of NO and MDA, associated with a significant decrease in antioxidant enzymes, especially CAT, were observed in the untreated

Table 1. Biochemical analysis and cardiovascular risk indices evaluation

Parameters	Groups				
	NC	STZ	STZ-CMS	STZ-CMS+INS	STZ-CMS+INS+PFC
Lipid profile					
Cholesterol (mg/dL)	65.95 ± 11.99	112.58 ± 18.90***	121.64 ± 21.73***	81.85 ± 11.70++/@@@	72.39 ± 15.42+++/@@@
Triglyceride (mg/dL)	75.52 ± 11.34	163.47 ± 23.98***	189.61 ± 29.49***/+	77.78 ± 11.89+++/@@@	68.52 ± 7.27+++/@@@
HDL-C (mg/dL)	25.20 ± 2.97	16.50 ± 2.27***	15.30 ± 1.34***	20.80 ± 2.53**/++/@@@	23.40 ± 2.17+++/@@@
LDL-C (mg/dL)	24.80 ± 5.03	40.10 ± 7.72***	36.90 ± 7.75**	28.10 ± 6.50++/@	26.10 ± 5.22+++/@@
VLDL-C (mg/dL)	15.10 ± 2.27	32.69 ± 4.80***	37.92 ± 5.90***/+	15.55 ± 2.38+++/@@@	13.70 ± 1.45+++/@@@
AIP	0.47 ± 0.10	0.99 ± 0.09***	1.08 ± 0.08***	0.57 ± 0.10+++/@@@	0.46 ± 0.06+++/@@@
CVRI-1	2.67 ± .070	6.87 ± 1.16***	8.04 ± 1.87***	3.98 ± 0.71+++/@@@	3.12 ± 0.80+++/@@@
CVRI-2	1.00 ± 0.25	2.47 ± 0.60***	2.42 ± 0.55***	1.37 ± 0.35+++/@@@	1.13 ± 0.26+++/@@@
Liver function					
AST (IU/L)	101.80 ± 8.78	224.90 ± 15.09***	242.40 ± 18.91***	132.00 ± 18.97**/+++/@@@	109.20 ± 11.70+++/@@@/$
ALT (IU/L)	65.70 ± 12.66	126.30 ± 14.12***	144.10 ± 13.89***/+	82.20 ± 12.29+++/@@@	70.50 ± 12.60+++/@@@
Renal function					
Creatinine (mg/L)	4.18 ± 0.33	7.13 ± 0.70***	8.01 ± 0.78***/+	5.11 ± 0.66*/+++/@@@	4.73 ± 0.47+++/@@@

AIP: Atherogenic index of plasma; CVRI: Cardiovascular risk indices; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; NC: Normal control; STZ: Streptozotocin; CMS: Chronic mild Stress; INS: Insulin; PFC: Phenolic fraction concentrates. Results are expressed as Mean ± SD (N=8-10). * P < 0.05, ** P < 0.01, *** P < .001 vs. normal control (NC). + P < 0.05, ++ P < 0.01, +++ P < 0.001 vs. untreated STZ-diabetic group. @ P < 0.05, @@ P < 0.01, @@@ P < 0.001 vs. untreated STZ-diabetic group exposed to CMS. $$ P < 0.01, $$$ P < 0.001 vs. STZ-diabetic group exposed to CMS and treated with insulin.
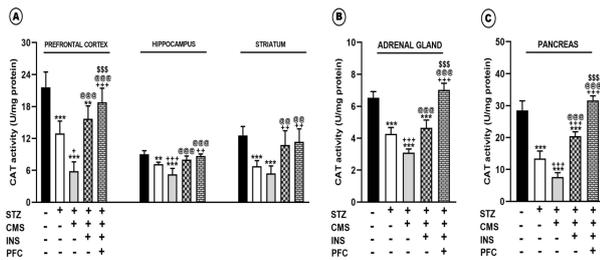
Figure 5. Catalase (CAT) activity in brain structures (A) and peripheral organs: Adrenal gland (B), Pancreas (C) in normal and STZ-diabetic rats exposed to chronic mild stress (CMS). Results are expressed as mean ± SD (N=6-8). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. normal control (NC). + $P < 0.05$, ++ $P < 0.01$, +++ $P < 0.001$ vs. untreated STZ-diabetic group. @ $P < 0.05$, @@p<0.01, @@@ $P < 0.001$ vs. untreated STZ-diabetic group exposed to CMS. $$ $P < 0.01$, $$$ $P < 0.001$ vs. STZ-diabetic group exposed to CMS and treated with insulin.

Figure 7. Malondialdehyde (MDA) level in brain structures (A) and peripheral organs: Adrenal gland (B), Pancreas (C) in normal and STZ-diabetic rats exposed to chronic mild stress (CMS). Results are expressed as Mean ± SD (N=6-8). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. normal control (NC). + $P < 0.05$, ++ $P < 0.01$, +++ $P < 0.001$ untreated STZ-diabetic group. @ $P < 0.05$, @@ $P < 0.01$, @@@ $P < 0.001$ vs. untreated STZ-diabetic group exposed to CMS. $$ $P < 0.01$, $$$ $P < 0.001$ vs. STZ-diabetic group exposed to CMS and treated with insulin.
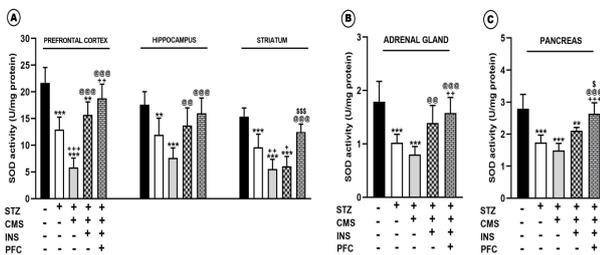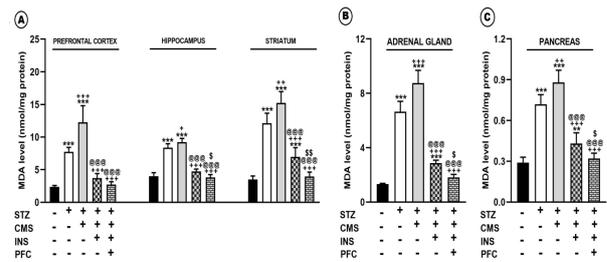

Figure 6. Superoxide dismutase (SOD) activity in brain structures (A) and peripheral organs: Adrenal gland (B) and Pancreas (C) in normal and STZ-diabetic rats exposed to chronic mild stress (CMS). Results are expressed as mean ± SD (N=6-8). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. normal control (NC). + $P < 0.05$, ++ $P < 0.01$, +++ $P < 0.001$ vs. untreated STZ-diabetic group. @ $P < 0.05$, @@ $P < 0.01$, @@@ $P < 0.001$ vs. untreated STZ-diabetic group exposed to CMS. $$ $P < 0.01$, $$$ $P < 0.001$ vs. STZ-diabetic group exposed to CMS and treated with insulin.
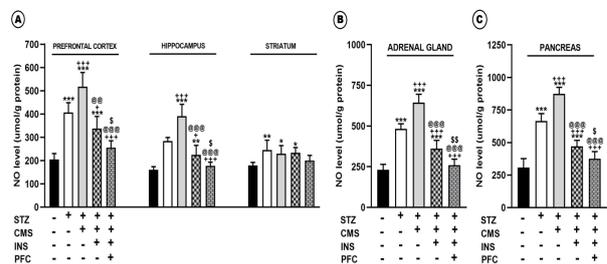

Figure 8. Nitrite oxide (NO) concentration in brain structures (A) and peripheral organs: Adrenal gland (B), Pancreas (C) in normal and STZ-diabetic rats exposed to chronic mild stress (CMS). Results are expressed as mean ± SD (N=6-8). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. normal control (NC). + $P < 0.05$, ++p<0.01, +++p<0.001 vs. untreated STZ-diabetic group. @ $P < 0.05$, @@ $P < 0.01$, @@@ $P < 0.001$ vs. untreated STZ-diabetic group exposed to CMS. $$ $P < 0.01$, $$$ $P < 0.001$ vs. STZ-diabetic group exposed to CMS and treated with insulin.

STZ-diabetic group exposed to CMS compared to the untreated STZ-diabetic group ($P < 0.05$). Compared to the untreated STZ-diabetic group exposed to CMS, treatment with insulin supplemented or/not supplemented with PFC for 12 weeks induced a significant reduction ($P < 0.01$) of MDA as well as NO, and significantly enhanced the activities of CAT and SOD ($P < 0.01$) in the treated STZ-diabetic groups. The PFC supplementation significantly regulated ($P < 0.05$) the MDA (except in hippocampus) and NO (except in striatum) products in the STZ-diabetic group exposed to CMS and treated with INS supplemented with PFC compared with the STZ-diabetic group exposed to CMS and treated only with INS. Also, CAT activity in all tissues (except in the hippocampus and striatum) increased significantly in the PFC-supplemented group compared to the STZ-diabetic group treated only with INS ($P < 0.001$).

Discussion

In spite of the significant advances for elucidating and understanding the physiopathology of T1D and stress,

as well as their complications, the proposed treatments are still very limited. It appears to be reasonable to assess whether supplementation in antioxidants can help prevent those complications. Therefore, the main objective of the current study was to investigate if insulin supplemented with PFC improves chronic hyperglycemia-related behavioral changes by mitigating oxidative stress in STZ-diabetic rats exposed to CMS.

In the present study, the STZ injection in adult rats resulted in a significant increase in FBG and corticosterone levels associated with a remarkable decrease in body weight. These findings overlap with other research conducted in STZ-diabetic rats (33). Moreover, the CMS paradigm used in the present study is the most commonly used model of depression (20). We found that continuous exposure of STZ-diabetic rats to CMS for 12 weeks increased the corticosterone level significantly, with a severe drop in body weight. Likewise, a previous animal study has shown that diabetic and non-diabetic rats, exposed to chronic restraint stress (4 h/day) for 6 weeks, have a significant increase in corticosterone level and a remarkable decrease

in body weight (33). It is known that stress stimulates the adrenal cortex to produce corticosterone in rodents via a hormonal cascade (34). The exact mechanism for this stimulation is still unclear, but it can be associated with altered activity of the central stress response system (the hypothalamic pituitary adrenal axis) (35). Consequently, the increase in corticosterone production during stress seems to be crucial for the defense system to be put into place. Several reports suggest that high concentrations of stress hormones could stop insulin-producing beta cells from working appropriately, which reduces the quantity of insulin they make (36). In the present study, treatment with insulin supplemented with PFC for 12 weeks significantly improved FBG and corticosterone levels and increased the body weight remarkably. The effects of PFC might be explained in terms of its anti-hyperglycemic action by inhibiting digestive enzymes and by its potential antioxidant activity (16). Previous reports demonstrated that coenzyme Q10, gallic acid, and caffeic acid, which are potent antioxidants, protected animals against chronic restraint stress-induced pathology (7,37,38).

Several experimental studies have indicated that exposure to either T1D or chronic stress, or both, induced abnormal lipid metabolism and dyslipidemia (32,39). Dyslipidemia has a crucial role in inducing diabetic heart pathology. This effect has been revealed via the evaluation of cardiovascular risk indices such as creatine kinase-MB, lactate dehydrogenase, troponin-I, CVRI-1, CVRI-2, and AIP (39). In the present study, continuous exposure of diabetic rats to CMS significantly increased lipid profile, atherogenic index, as well as cardiovascular risk index 1 and 2. Under chronic stress circumstances such as living with a chronic illness, increased corticosteroids can induce insulin resistance, which promotes triglyceride synthesis and can retard the clearance of high-density and low-density lipoproteins (40). In fact, dietary phenolic compound consumption may prevent the development of cardiovascular diseases by decreasing blood lipid profile (41). Therefore, the treatment of stressed diabetic rats with INS supplemented with PFC for 12 weeks remarkably improved blood lipids and reduced cardiovascular risk indices. Furthermore, several experimental and clinical data have revealed that phenolic compounds exert their effects on lipid metabolism via upregulating LDL-C receptors, reducing the absorption of intestinal cholesterol, and controlling apolipoprotein-A1 expression, which stimulates the pathway of reverse cholesterol (42,43). We hypothesize that the anti-hyperlipidemic effect of PFC in stressed diabetic rats might be related to its increased amounts of phenolic compounds viz. caffeic, gallic, dihydroxybenzoic, and chlorogenic acids (16).

The presence of hyperglycemia-related hyperlipidemia is linked with increased ROS production, which promotes oxidative damage in several organs (44). Chronic stress can alter the physiological activity of HPA axis, which leads to an elevated production of ROS (45). For these reasons,

one of the main objectives of the present study was to assess the long-term complications of both hyperglycemia and CMS on oxidative stress markers, especially in brain regions, in order to evaluate the neuroprotective effects of insulin supplemented with PFC. Previous researches suggest that changes in the physiological functioning of the antioxidant defense system are among the principal causes of diabetic neuropathogenesis due to the disequilibrium between antioxidants and free radicals (46). Increased amounts of oxidative stress agents in nerve cells have been revealed to activate neuronal alteration by peroxidation of unsaturated lipids (lipid peroxidation). This latter is the major process responsible for nerve cell damage by ROS, which destroys neuronal structural integrity in the different brain areas of diabetic rats (47). In the present study, we have demonstrated that the association of two different stressors (T1D and CMS) significantly increased the oxidative stress products and decreased the significant effect of antioxidant enzymes, especially in brain regions. In line with our results, previous experimental research with the animal model of CMS demonstrated that this model induced a decrease in SOD activity in prefrontal cortex, striatum, and hippocampus accompanied with an increase in protein peroxidation as well as CAT activity in the prefrontal cortex and the hippocampus (48). An animal model of repeated restraint stress revealed a significant elevation in lipid peroxidation levels in the hippocampus (49). The findings of our analysis revealed that insulin therapy significantly reduced oxidative stress by restoration of SOD and CAT and suppression of MDA and NO products induced by T1D combined with CMS in adrenal glands, pancreas, hippocampus, and prefrontal cortex. Insulin can be a powerful antioxidant product for the treatment and prevention of oxidative-stress-damage-induced diabetic neuropathy through the regulation of nuclear factor E2-related factor 2 signaling pathway (50). Insulin treatment supplemented with PFC significantly protected the STZ-diabetic rats exposed to CMS for 12 weeks from oxidative stress products compared to the STZ-diabetic group exposed to CMS and treated with only insulin. Thus, this therapy of chronic administration of insulin supplemented with PFC enhanced antioxidant defenses and reduced oxidative stress damage in the brain of STZ-diabetic rats exposed to CMS. Our results revealed that PFC might attenuate brain injury in stressed diabetic rats through the attenuation of oxidative stress. This potential feature might be attributed to the increased quantity of its phenolic compounds (16).

The behavioral alterations induced by chronic hyperglycemia are associated with numerous factors, including reduced monoamine-neurotransmitters concentrations such as serotonin in many brain regions (9). Likewise, prolonged corticosterone exposure decreases serotonin concentration in different brain regions, which is the crucial cause of psychiatric disorders (51). Moreover, the prolonged release of corticosterone

in the hyperglycemic state results in prolonged exposure of neurons to corticosterone, causing dendritic atrophy, decreased synaptic plasticity, and reduced neurogenesis, leading to neuronal damage, which in turn promotes neuropsychiatric disorders (13,14).

This is the first study that revealed the neuroprotective effect of insulin-supplemented PFC on the STZ rat model of diabetes. We demonstrated that this treatment has anti-anhedonic, anxiolytic, and antidepressant effects, as evidenced by improving hyperglycemia- and CMS-induced behavioral alterations in various paradigms (SPT, EPM, OFT, and FST).

Chronic mild stress, especially when associated with diabetes, can contribute to enhanced oxidative damage in CNS, which can lead to behavioral changes such as anxiety and depression (32). In the current study, the untreated STZ-diabetic rats exposed or not exposed to CMS for 12 weeks exhibited anxiety-related behavior. This is in agreement with the previous investigations of anxiety-related behavior in animal models of diabetes induced by STZ-treatment (1,52). Insulin supplemented with PFC had more significant effects on anxiety than only insulin treatment in STZ-diabetic rats exposed to CMS, as revealed by the decreased symptoms assessed using the OFT and EPM tests. This anxiolytic-like effect of insulin has previously been reported in STZ-diabetic rats (53). The current study is the first report that shows this treatment combination has these effects on STZ rat model of diabetes under stress conditions. In this context, it has been documented that chronic stress enhanced release of hippocampal serotonin in animals that exhibit high levels of anxiety (54). Continuous exposure of STZ-diabetic rats to CMS for 12 weeks increased the corticosterone level significantly. This increased corticosterone concentration can trigger anxiety-related behaviors in rodents by various mechanisms (55), which let us suggest the possibility of causing dysfunction in serotonin release. An experimental study investigating the effect of phenolic compounds on corticosterone level and some neurotransmitters reported that phenolic and flavonoid derivatives promote the release of noradrenaline and serotonin levels, respectively (56). The anxiolytic effect of PFC might be attributable to favoring the stimulation of the central serotonin system by improving its availability in the different brain regions and/or by mitigating oxidative stress in different brain regions.

Chronic stress and T1D can contribute to other neuropsychiatric disorders such as anhedonia- and depressive-like behaviors (57,58). In this study, STZ-induced T1D in rats decreased remarkably the percentage of sucrose preference as observed in the SPT. In accordance with these findings, an experimental study has noted that T1D induced significant changes in sucrose preference as indicators of anhedonia-like behavior (58). However, this study highlighted that long-term hyperglycemia in rats induced a remarkable decrease of Fos expression in the lateral septal nucleus. In addition, an abnormality in this nucleus revealed by its low neuronal activity has been linked to anhedonia-like behavior (59). Regarding CMS effects on behavior, the results revealed that 12 weeks of exposure to CMS increased significantly anhedonia-like behavior. In this line, a clinical study with functional magnetic resonance imaging revealed that anhedonia severity was associated with a reduction in the striatum volume, suggesting that the striatum plays a significant role in the neuro-pathogenesis of depression (60). Similar results were reported in another study, which demonstrated a significant correlation between CMS-induced anhedonia-like behavior and 5-hydroxytryptamine expression in the hippocampus (61). In the present study, insulin treatment or supplemented with PFC protected the rats remarkably against anhedonia-like behavior induced by diabetes associated with stress.

Anhedonia was reported as a major symptom of depression. In the current study, CMS significantly increased the depressive-like behavior on diabetic rats when compared to diabetic rats without stress conditions. Similar findings were reported in hyperglycemic rats exposed to stress (4 h/day for 6 weeks) (32). Additionally, our results are coherent with another work undertaken by Huynh et al (62), who reported that chronic stress could induce aggressiveness and increase depressive-like behavior. Nevertheless, other works have not revealed such behavioral changes after chronic stress (63). One reason for these findings could be explained by the experimental procedures and the total duration of stress exposition. In this line, antidepressant-like effects of insulin were previously reported in STZ-diabetics rats (21,53,64). Contrariwise, our results revealed that stressed diabetic rats treated with insulin were less likely to decrease the immobility time in FST, indicating the absence of any significant antidepressant effect of insulin in this model of rats. Probably, insulin antidepressant effects do not occur when diabetic rats are under stress. However, the treatment with insulin combined with PFC significantly decreased the immobility time in the FTS when compared to STZ-diabetic rats exposed to CMS treated only with insulin. Oxidative stress damage, especially in the prefrontal cortex and hippocampus, was associated with behavior alteration in rats with chronic hyperglycemia and/or under stress conditions (21,65). Our findings are in agreement with numerous lines of evidence that highlight oxidative stress damage plays a crucial role in the development of depressive-like behavior (66). Further, this is the first study revealing that daily treatment with insulin combined with PFC was able to protect the rats against depressive-like behaviors induced by diabetes and reinforced by CMS. This result showed that PFC might attenuate brain injury in stressed diabetic rats through the suppression of oxidative stress in the hippocampus and prefrontal cortex. This potential propriety might be attributed to the increased quantity of its phenolic

compounds, especially phenolic acids such as caffeic acid (16). Cumulative evidence summarized in a recent review showed that phenolic compounds, inhibiting the pathway of MAPK signaling, mediated oxidative damage in depression (67).

Conclusion

In summary, the current work is the first to elucidate the preferable antioxidant effect of the insulin associated with PFC over insulin alone. We showed that this treatment decreased oxidative stress, especially in brain regions in stressed diabetic rats. This combination was also linked with a significant effect in modeling the corticosterone level as well as glucose homeostasis and lipid parameters, which are markedly altered in T1D associated with stress. Besides, our findings showed that the associated treatment possesses important anxiolytic and antidepressant-like proprieties in this rat model, which can be mainly mediated by its capacity to protect brain cells against ROS triggered by diabetes and/or chronic stress or both. Nevertheless, more research is needed to identify the mechanisms behind these anxiolytic and antidepressant effects of PFC and extend these results before safe employment in humans.

Authors' contributions

BS and YA invented the work's conception, performed the experiments and wrote the draft; TA, HA, and NF participated in the study design and helped in manuscript preparation; BH helped in statistical analysis and critical revision of the manuscript; AA performed the revision of the manuscript. All authors read and approved the final manuscript.

Conflict of interests

All authors of this work certify that they have no conflict of interest.

Ethical considerations

All experimental procedures were performed under the current ethical and care guidelines for the care of laboratory animals and were approved by the Animal Ethics Committee of Biology and Health laboratory, Faculty of science, Ibn Tofail University, Kenitra, Morocco (NUT/00/91, dated June 2021).

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