



Phenolic fraction concentrates supplementation ameliorates learning and memory impairments in chronically stressed streptozotocin-diabetic rats by reducing brain tumor necrosis factor- α

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ABSTRACT

Introduction: The present work aims to assess if insulin combined with phenolic fraction concentrates (PFCs) prevents diabetes-related cognitive impairments by controlling neuroinflammation in streptozotocin-induced diabetic rats exposed to chronic mild stress (CMS).

Methods: Directly after confirming the hyperglycemia, diabetic animals were treated with insulin combined with PFC and were exposed to 2 stressors/day for 12 weeks. Then, four cognitive tests were carried out to assess learning and memory performances. Finally, the rats were anesthetized, blood samples were collected for corticosterone and Tumor necrosis factor alpha (TNF- α) analysis, and the brain regions viz. striatum, hippocampus, and prefrontal-cortex of each hemisphere were dissected out for TNF- α analysis.

Results: Both diabetes and stress could induce learning and memory impairments, which were more prominent in stressed diabetic animals, and significantly reversed by insulin treatment supplemented with PFC compared to the insulin monotherapy. Moreover, diabetic rats exposed to CMS displayed disturbances in glucose homeostasis as well as corticosterone secretion. These dysfunctions were linked to the significant increase of TNF- α in the blood as well as in the prefrontal cortex, hippocampus, and striatum. Insulin significantly ameliorated this inflammatory abnormality, while the supplemented treatment showed a significant effect, by stabilizing TNF- α to its normal levels in the hippocampus and in the blood when compared to insulin monotherapy.

Conclusion: Insulin supplemented with PFC has a favorable effect over insulin alone on inflammatory aberrations linked with type 1 diabetes and stress in animals, confirming the preference of the combined treatment over insulin for the management of cognitive impairment in stressed diabetic subjects

Implication for health policy/practice/research/medical education:

The present study is the first to enlighten the anti-inflammatory action of the insulin treatment supplemented with phenolic fraction concentrates (PCFs). This combination treatment revealed a favorable effect over insulin alone on inflammatory aberrations associated with type-1 diabetes and stress in rats. This treatment markedly protected these rats against learning and memory impairment, which indicates its capacity to protect brain cells against inflammation, triggered by stress and diabetes.

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Introduction

Type 1 diabetes mellitus (T1DM) is a metabolic disease generally caused by autoimmune-induced destruction of the pancreatic beta cells responsible for the secretion of insulin, which regulates the levels of blood glucose in the body (1,2). The loss of insulin secretion results in the body's inability to control blood glucose concentrations, which in turn promotes severe complications in peripheral as well as central organs (3). According to the latest International Diabetes Federation estimates, more than 537 million adults are living with diabetes, and this number is predicted to increase to 783 million by 2045 (4).

Worldwide, the concurrence of T1DM and cognitive decline and/or dementia constitutes a crucial health problem among people with diabetes, whose risk of dementia is 56%-73% greater than non-diabetic people (5). Furthermore, cumulative evidence summarized in a recent mini-review revealed that the relationship between dementia and T1DM was stronger than that with Alzheimer disease (6). Moreover, in a study conducted by Ohara et al, it was demonstrated that even glucose intolerance significantly elevates the risk of dementia and Alzheimer's disease risk (7). In this context, several biological mechanisms suggest that hyperglycemia may elevate the risk of dementia. These include brain insulin resistance, overproduction of advanced glycation end-products, and reduced insulin signaling, which reduces the synthesis of insulin-degrading enzyme (a principal regulator of amyloid β -protein levels in neuronal and microglial cells) (8). In animal models, it has been established that diabetes induces changes in neural plasticity in critical brain areas, viz. prefrontal cortex and hippocampus (9). Brain plasticity can include neuroanatomical damage such as changes in neuronal dendritic morphology, impairment of long-term hippocampal potentiation, and neurochemical profile alterations (9). Eventually, these alterations can significantly contribute to the development and progression of impairments in learning and memory in diabetics.

Living with a chronic health condition such as type 1 diabetes is challenging as it interferes with social, mental, and physical functions, which affect the quality of life of these patients (10). It is well known that stress is a significant contributor to physiological disturbances in humans. Furthermore, hormonal changes that occur during stress may markedly disturb glucose, particularly in individuals with diabetes (11). A previous work suggests that high concentrations of corticosterone could be deleterious to beta cells that produce insulin (12). It has been shown that chronic stress is associated with significant changes in physical variations in neuronal networks. Also, several experimental studies have demonstrated stress-related impacts on the prefrontal cortex as well as the limbic system, including the hippocampus. These impacts are characterized by reductions in the volume of the respective brain structures, as well as alterations in neuroplasticity

due to reduced spine density and dendritic atrophy (13). In regards to hippocampal long-term potentiation impairment, exposure to stress impairs learning and memory (14).

Several studies have revealed a significant relationship between inflammation, diabetes, and chronic stress (15,16). It is known that chronic hyperglycemia induces pro-inflammatory cytokine production as a result of increased reactive oxygen species levels (17). Additionally, chronic stress can change the activity of the hypothalamic-pituitary-adrenal axis, which induces overproduction of these radicals (18). Being exposed to diabetes and stress can induce pathophysiological changes (e.g. inflammation) in the brain, and these alterations can be manifested as learning and memory deterioration (19). The overproduction of proinflammatory cytokines such as tumor necrosis factor- α (TNF- α) becomes crucial and leads to brain cell death and impaired brain plasticity. Consequently, this promotes learning and memory impairments (20). Strategies to reduce the overproduction of inflammatory cytokines might have significant neuroprotective effects in diabetes (21).

Natural antioxidants, including phenolic compounds, have been known to control various diseases linked with inflammation of the nervous tissue through anti-inflammatory and antioxidant properties (22). Therapy with antioxidant compounds that attenuate oxidative stress as well as neuroinflammation may serve to reduce diabetes complications such as cognitive impairment (23). Accordingly, date seeds present a rich source of phenolic compounds with a range of significant biological activities (24). In addition to having antioxidant and antidiabetic activities (25), date seeds are also considered a promising anti-inflammatory product. Date seed extract has shown to be an anti-inflammatory agent due to its ability to control inflammatory mediators such as interleukin (IL)-1, IL-2, IL-6, interferon-gamma (IFN- γ) and TNF- α (26), and suppress the nuclear factor kappa B (NF- κ B) translocation. Date seeds exhibit a significant anti-inflammatory effect by downregulating the expression of cyclooxygenase (COX)-1, cyclooxygenase-2, transforming growth factor-beta (TGF- β), and IL-1 β (27).

To our knowledge, there are no studies that have explored the effect of phenolic fraction concentrates (PFCs) as a rich product source of antioxidant compounds on brain inflammation induced by T1DM and stress. The main goal of the present study was to investigate the neuroprotective effect of chronic treatment with insulin supplemented with PFCs on learning and memory performances and TNF- α level in the brain areas and blood of diabetic rats exposed to chronic mild stress (CMS).

Material and Methods

Plant material

Fresh fruits were harvested from the date palm tree (*Phoenix dactylifera* L.) grown in Ouarzazate region (30°55'23"

N, 6°54'15" W). Plant samples were authenticated and identified by Professor Youssef Aboussaleh, Head of the Applied Nutrition and Neurosciences Research Unit, Department of Biology, Faculty of Sciences, Ibn Tofail University, Kenitra, Morocco. A specimen was then deposited at the university herbarium under number: DASE-215/2020. Collected dates were pitted and seeds were removed and washed with tap water, then dried and stored under constant darkness until use.

Preparation of phenolic fraction concentrates

The PFC extraction of the date seeds was performed as described in our previous study (25). Date seeds were washed with tap water, let dry at 50°C in the oven for 48 hours, and ground until the obtention of seeds powder of 1-1.5 mm diameter. Then, to dissolve the phenolic compounds, the resulting powder was macerated with 50% acetone and 2% acetic acid in a solvent/sample (10:1) ratio. The mixture was homogenized using a magnetic stirring plate timed at a stirring speed of 120 rpm and 45°C for one hour and was then centrifuged at room temperature and filtered on Whatman No. 4 filter paper to remove the particles. The obtained clear filtrate was evaporated at 60°C until dryness using a rotary evaporator (Buchi Rotavapor R-300 V). The phenolic compounds were purified with N-butanol using a separatory funnel and the resulting fraction was then evaporated at 60°C. Finally, the phenolic fraction produced put to dryness at 60°C in the oven and ground into powder.

Animals

Forty adult male Wistar rats weighing (300-320 g) were used for this experiment. All rats were obtained from the animal breeding house of Ibn Tofail University (Kenitra, Morocco). They were then transferred to the experimental room and housed in standard plexiglass cages (430 × 290 × 210 mm) and allowed to acclimatize to the controlled experimental room conditions and free access to standard food and tap water.

Experimental diabetes induction

In the present study, we used the streptozotocin (STZ)-induced type 1 diabetic rat model. In our experiment, we used a single dose of 65 mg/kg STZ (Sigma, St Louis, MO, USA) to induce diabetes following the procedure described by Furman (28). Overnight fasting rats received an intraperitoneal (i.p.) injection of 65 mg/kg STZ dissolved in 0.1 mol/L sodium citrate buffer at pH=4.5. Directly after STZ administration, animals were given aqueous glucose (5%) for 12 hours to avoid hypoglycemia. Three days post-injection, fasting blood glucose (FBG) was measured to confirm the hyperglycemia. Blood samples were collected using the tail prick method. Only rats with blood glucose levels greater than or equivalent to 250 mg/dL were considered diabetic and were included in the experiment.

Chronic mild stress procedure

The CMS paradigm was preceded as described previously by Zhang et al (29), with slight modifications for this study. In this CMS strategy, animals were exposed to a sequence of mild stressors, including cold stress (4°C, 1 hour), water deprivation (24 hours), tilted cage (45°C, 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). Out of these ten mild stressors, STZ-rats were exposed randomly to two unpredictable stressors daily (6 days/week) starting 8:00 AM over 12 weeks. To avoid habituation, the same stressor was not presented again to the rats for 3 consecutive days.

Experimental design

Upon transfer to the experimental room, animals were left undisturbed for acclimatization and were then gently handled for three consecutive days. Rats (n=40) were randomly assigned into five experimental groups and divided as follows: (1) Control (n=8); in this group, rats were maintained normal and did not receive any treatment; (2) STZ Control (n=8), which was the untreated STZ-induced diabetic group serving as the diabetic control; (3) STZ+CSM (n=8), which was the untreated STZ-induced diabetic group exposed to CMS. Each rat in these two groups received saline (1 mL 0.9% NaCl /200 g/d); (4) STZ+CMS+INS (n=8), which was the STZ-induced diabetic group exposed to stress and treated with subcutaneous injection of insulin (6 UI/day; s.c.) and finally, (5) STZ+CMS+INS+PFC (n=8), which was the STZ-induced diabetic group exposed to CMS and treated with insulin (6 UI/day; s.c.) supplemented with the phenolic fractions concentrates (50 mg/kg dissolved in 1 mL 0.9% NaCl/200 g per dose). The dose of insulin administered was divided into 2 separate injections: 2 UI in the morning and 4 UI in the afternoon, based on a previous study conducted by De Moraes et al (30). However, the PFC administration protocol used (50 mg/kg; once/day; per os), was based on our recent findings that the PFC showed in vitro and in vivo antioxidant and anti-hyperglycemic activities in addition to its safety for oral administration as evidenced by the acute and subacute oral toxicity studies conducted in vivo (25). Thus, the dose was regularly adjusted based on the body weight changes.

Cognitive tests

All behavioral tests were carried out in a quiet room under appropriate illumination. All animals were exposed to the elevated plus maze, open field, then the Y maze, and the Morris water maze (MWM) tests to assess learning and memory performances.

Elevated plus maze test

The elevated plus-maze test was used to evaluate the

degree of spatial long-term memory as described earlier (31). The test apparatus consisted of four arms of equal sizes consisting of two wooden “open” arms (50 cm × 10 cm) that were opposite to each other and crossed with two “closed” arms opposite to each other and enclosed by 40 cm height walls. At the beginning of the acquisition session, each rat was gently placed at the distal end of an open arm at the edge of the apparatus facing the floor. We recorded transfer latency, which was the time required for the animal to move from the open arm to one of the enclosed arms. We consider a successful entry when the animal crossed the imaginary line separating the closed arm from the central platform with all his four paws. Rats that did not enter to a closed arm within 90 seconds were excluded from the experiment. Then, after entering the closed arm, the rat was allowed to explore the maze freely regardless of open or closed arms for 10 seconds. The retention session was performed 24 hours after the acquisition session and the transfer latency was recorded for all rats.

Morris water maze test

The MWM test was used to assess spatial learning and reference memory as described by Morris et al (32), with slight modifications for this study. MWM was originally designed to assess the rat's ability to learn to navigate to a specific location in a relatively large spatial environment. The water maze consisted of a round tank (120 cm in diameter and 40 cm in depth) filled with tap water at 30 cm of height with the temperature maintained at 22°C. The tank was divided into four imaginary quadrants (North, South, West, and East), each including a visual cue outside the surface of the water on the distal part of the wall of the maze. A hidden platform (11 × 14 cm) submerged 2 cm under the water surface was located in the middle of one of the four imaginary quadrants of the pool and maintained in the same position during all trials. The training session consisted of five consecutive trials during which the animals were repeatedly placed into the tank facing the wall and given 60 seconds to learn to escape by locating the hidden platform, each trial starting from a different starting position. The animal who escaped to the platform was let on it for 10 seconds and the animal who failed to locate the escape platform in 90 seconds was gently guided to it. The test session was similar to the training session, and the animal performance was videotaped by a video camera placed on the ceiling above the maze. The latency to escape to the platform was calculated, and it is the time spent by the rat to find the platform rendered invisible by making the water opaque.

Recognition memory test

The Novel object recognition test (NORT) was used to investigate aspects of learning and memory in rats as described previously by N'Go et al (33). The test was performed in an open box of 50 cm in length, 50 cm in

width, and 40 cm in height, and it was completed over 3 days: the first day for habituation, the second day for training and short-term recognition (STM) memory testing, and the third day for long-term recognition (LTM) memory testing. During habituation rats were allowed to freely explore the arena of the open box for 5 minutes. During the training day, they were allowed to explore the arena with two identical objects for 5 minutes and then returned to their home cage. For short-term memory assessment, rats were tested just 2 hours after the training session with a novel object placed with a familiar object. Finally, for long-term memory assessment, rats were tested 24 hours after the training session with another novel object different to that of the short-term memory testing and placed with the familiar object. The experiment was videotaped and the following parameters were measured: The ratio of the preference for the novel object (calculated as the frequency of exploration of the novel object divided by the total frequency of exploring both objects) and the exploration behavior, which was measured as the time when the rat was directing its nose at a distance less than or equal to 3 cm from the object without touching it.

Y-maze test

The Y maze test was used to evaluate spatial working memory by measuring spontaneous alternations as described previously (34). The maze was made of wood and it consisted of three identical arms labeled randomly as A, B, and C arms with an angle of 120° between each of the two arms. Each was painted in different color patterns and measured 40 cm × 10 cm × 13 cm (length × width × height). At the start of the testing session, each rat was allowed to freely explore all three arms of the maze during 8 minutes and the session was recorded with a video camera placed above the apparatus. Video recordings were later analyzed and the percentage of spontaneous alternation was calculated using this equation: %Alternation = $100 \times (\text{Number of alternation} / \text{Total arm entries} - 2)$. This behavior was driven by an innate curiosity of rodents to explore previously unvisited areas.

Preparation of homogenates

At the end of behavioral tests, the rats were deeply anesthetized with chloral hydrate (100 mg/kg) and then sacrificed by cervical dislocation. Blood samples were collected by cardiac puncture from each animal for serum biochemical measurements. Finally, the brain regions of each hemisphere (striatum, hippocampus and prefrontal cortex) were quickly dissected on ice, frozen by liquid nitrogen, and stored at -80°C until the analysis of TNF- α content. 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 (14000 g), and then stored at -80°C for the subsequent ELISA assays as previously described by Benmhammed et al (35).

Biochemical assays

Plasma corticosterone assay

A separate frozen sample aliquot from each animal was used for each assay and plasma corticosterone levels were evaluated using commercial rat ELISA kits according to each manufacturer's instructions. Thus samples were brought and kept on ice to be used immediately. We measured the absorbance at 450 nm using the microplate reader, and the plasmatic corticosterone value was expressed in ng/mL.

TNF- α assay

TNF- α levels were measured using a commercial rat TNF- α ELISA kit according to the manufacturer's protocols (KRC3011; Invitrogen). The TNF- α level in tissue homogenates was expressed as picograms of cytokine per g of proteins and expressed as picograms of cytokine per millilitre in blood serum.

Protein assay

The concentration of total protein in samples was measured using the Bradford protein assay. Protein levels were determined using bovine serum albumin (0 to 15 mg/mL) used as a standard, as described by Bradford (36), in accordance with the manufacturer's protocols (E530 Biotechnology).

Statistical Analysis

All statistical analyses were performed using SPSS software for Windows (version 26). The data were expressed as mean \pm standard deviation (SD). The experimental values were compared to their corresponding control. Parametric data were analyzed using one-way and two-way ANOVA analysis of variance to illustrate the significant difference between the experimental and control groups, followed by

Tukey's multiple comparison test. Statistical significance was assumed at $P < 0.05$ unless otherwise noted.

Results

Effects of insulin combined with PFC on FBG and corticosterone levels in chronically stressed diabetic rats

Fasting blood glucose variations

As depicted in Figure 1, induction of T1D (T₀) produced the disturbance of homeostasis of glucose in Wistar rats manifested by a highly significant increase of FBG ($P < 0.001$). Exposure to CMS for 12 weeks reinforced significantly FBG level elevation in diabetic rats ($P < 0.01$) in contrast to unstressed diabetic rats. Compared to untreated diabetic rats exposed to CMS, blood glucose was improved gradually at the end of treatment in treated diabetic rats exposed to CMS ($P < 0.001$). Insulin monotherapy significantly reversed STZ-CMS-induced variations in glucose homeostasis. The combination treatment possessed a significant effect, stabilizing glycemia to its normal levels ($P < 0.001$) when compared to diabetic rats treated only with insulin.

Corticosterone level changes

As shown in Figure 2, the diabetic group revealed a significant increase in corticosterone level ($P < 0.001$) compared to the normal control group at the end of treatment. Exposure to CMS for 12 weeks induced a remarkable increase ($P < 0.001$) in corticosterone levels in diabetic rats in contrast to unstressed diabetic rats. Insulin monotherapy or combined with PFCs significantly decreased corticosterone levels in chronically stressed diabetic rats ($P < 0.001$) compared to untreated diabetic rats. The combination treatment possessed a significant effect, stabilizing corticosterone to its normal levels ($P < 0.001$) compared to diabetic rats treated only with insulin.

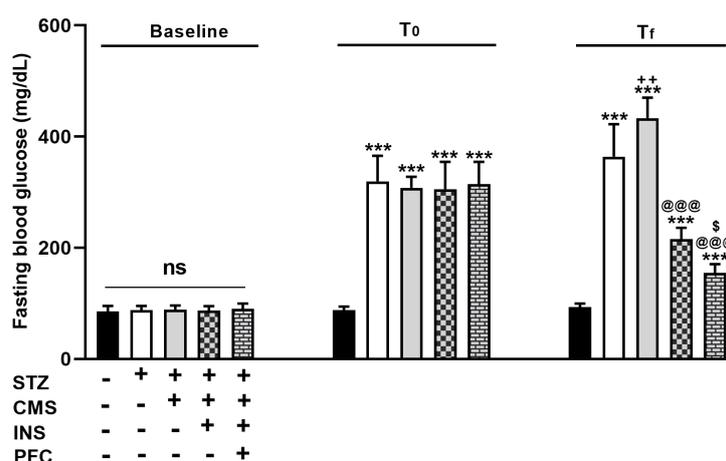


Figure 1. Fasting blood glucose (FBG) changes in normal and streptozotocin (STZ)-induced diabetic rats exposed to chronic mild stress (CMS) three times; baseline: before STZ injection, T₁: hyperglycemia confirmation day, and T₂: end of study "12 weeks". Results are presented as mean \pm SD (N=8). *** $P < 0.001$ vs. normal control group. ** $P < 0.01$ vs. untreated STZ-induced diabetic group. @@@ $P < 0.001$ vs. untreated STZ-induced diabetic groups exposed to CMS. § $P < 0.05$ vs. STZ-induced diabetic group exposed to CMS and treated only with insulin (one-way ANOVA followed by Tukey's multiple comparison test).

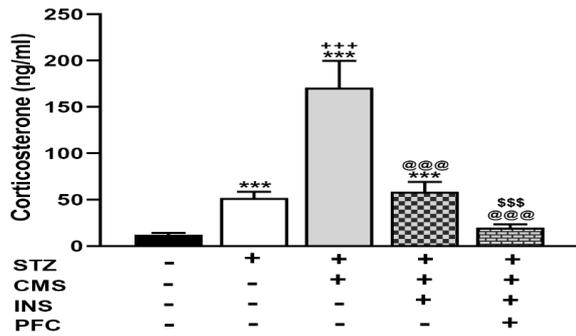


Figure 2. Corticosterone levels in normal and streptozotocin (STZ)-induced diabetic rats exposed to chronic mild stress (CMS) at the end of the study (12 weeks of treatment). Results are presented as Mean \pm SD (N=8). *** P < 0.001 vs. normal control group. +++ P < 0.001 vs. untreated STZ-induced diabetic group. @@@ P < 0.001 vs. untreated STZ-induced diabetic groups exposed to CMS. \$\$\$ P < 0.001 vs. STZ-induced diabetic group exposed to CMS and treated only with insulin (one-way ANOVA followed by Tukey's multiple comparison test).

Effects of insulin combined with PFC on cognitive abilities in chronically stressed diabetic rats

Short- and long-term recognition memory in recognition memory test

The percentage of recognition index during STM- and LTM-recognition phases significantly varied between the groups (Figure 3). As shown in this figure, the untreated diabetic group revealed a significant decrease in the STM (Figure 3A), as well as in the LTM (Figure 3B) index (%) compared to the normal control group (P < 0.001). Untreated diabetic rats exposed to CMS showed the lowest STM- and LTM-recognition index when compared to untreated diabetic group (P < 0.001). However, insulin treatment improved significantly short-term (P < 0.001) and long-term (P < 0.01) recognition memory deficit observed in the untreated diabetic rats exposed to CMS. The combination treatment significantly protected STZ-CMS-induced STM and LTM-recognition impairment

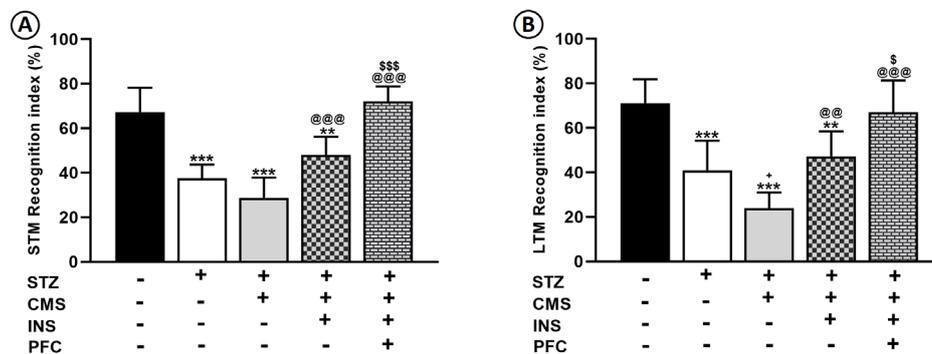


Figure 3. Effects of chronic treatment of insulin combined with phenolic fraction concentrate (12 weeks) on short- (A) and long- (B) term recognition memory in normal and streptozotocin (STZ)-induced diabetic rats exposed to chronic mild stress (CMS). Results are presented as mean \pm SD (N=8). ** P < 0.01, *** P < 0.001 vs. normal control group. + P < 0.05 vs. untreated STZ-induced diabetic group. @@@ P < 0.01, @@@ P < 0.001 vs. untreated STZ-induced diabetic groups exposed to CMS. \$\$\$ P < 0.05, \$\$\$ P < 0.001 vs. STZ-induced diabetic group exposed to CMS and treated only with insulin (one-way ANOVA followed by Tukey's multiple comparison test).

(P < 0.001; P < 0.05), respectively, compared to the treatment only with insulin.

Spatial working memory in Y maze test

In this study, the Y maze test was used to assess the spatial working memory capacities, and the results obtained are presented in Figure 4. Statistical analysis demonstrated a significant difference in the correction alteration (%) between the normal control group and diabetic group (P < 0.001), untreated stressed diabetic group (P < 0.001), and stressed diabetic group treated with insulin (P < 0.001). Exposure to CMS for 12 weeks significantly decreased the percentage of correction alteration in diabetic rats (P < 0.05) in contrast to unstressed diabetic rats. Nevertheless, the treatment of chronically stressed diabetic rats with insulin significantly enhanced the correction alteration (%) (P < 0.001) when compared to the untreated stressed diabetic group. The chronically stressed diabetic rats treated with insulin combined with phenolic fractions concentrate for 12 weeks performed significantly better in the Y-maze test compared to the rats treated only with insulin (P < 0.01).

Spatial long-term memory in elevated plus maze test

The effects of chronic treatment of insulin only or combined with phenolic fraction concentrate on the performance of spatial long-term memory acquisition and retention (mean transfer latencies) in normal and STZ-induced diabetic rats exposed to CMS are summarized in Figure 5. Statistical analysis showed that STZ-induced T1D had no effect on the transfer latencies (acquisition phase) compared to that of the control normal group (P > 0.05). Chronic exposure to CMS significantly increased the transfer latency of the acquisition trial in diabetic rats (P < 0.05) in contrast to unstressed diabetic rats. Post hoc test showed that chronic treatment with insulin combined with phenolic fraction concentrate significantly decreased transfer latency on acquisition trial

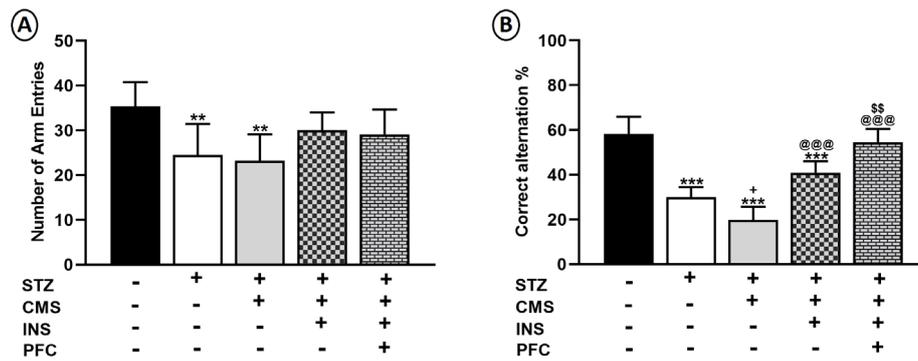


Figure 4. Effects of chronic treatment of insulin combined with phenolic fraction concentrate (12 weeks) on spatial working memory in normal and streptozotocin (STZ)-induced diabetic rats exposed to chronic mild stress (CMS). A: The number of arm entries, B: % of correct alternation. Results are presented as mean \pm SD (N=8). ** $P < 0.01$, *** $P < 0.001$ vs. normal control group. + $P < 0.05$ vs. untreated STZ-induced diabetic group. @@@ $P < 0.001$ vs. untreated STZ-induced diabetic groups exposed to CMS. \$\$\$ $P < 0.01$ vs. STZ-induced diabetic group exposed to CMS and treated only with insulin (one-way ANOVA followed by Tukey's multiple comparison test).

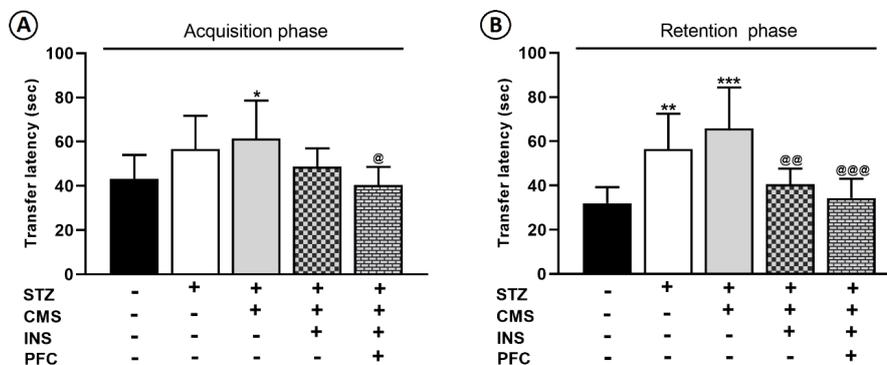


Figure 5. Effects of chronic treatment of insulin combined with phenolic fraction concentrate (12 weeks) on the performance of spatial long-term memory acquisition (A) and retention (B) in normal and streptozotocin (STZ)-induced diabetic rats exposed to chronic mild stress (CMS). Results are presented as mean \pm SD (N=8). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. normal control group. @ $P < 0.05$, @@ $P < 0.01$, @@@ $P < 0.001$ vs. untreated STZ-induced diabetic groups exposed to CMS (one-way ANOVA followed by Tukey's multiple comparison test).

($P < 0.05$) as compared to stressed diabetic rats without treatment. However, the Post hoc test revealed that STZ-induced T1D had a significant effect on transfer latency ($P < 0.01$) evaluated on retention trial (day 2). Also, exposure to CMS for 12 weeks significantly increased the transfer latency in diabetic rats ($P < 0.001$) in contrast to unstressed diabetic rats. Further, chronic treatment with insulin only or insulin combined with phenolic fraction concentrate significantly decreased the transfer latency in the retention trial ($P < 0.01$; $P < 0.001$), respectively, as compared to the stressed diabetic rats without treatment.

Spatial learning and reference memory in MWM test

At the end of treatment, spatial learning and reference memory functions were evaluated in the MWM test, and the results obtained are presented in Figure 6. The escape latency time for the trained animals decreased significantly over the course of all learning trials in all studied groups (Figure 6A). Actually, on the 1st

training day, there was no notable difference among the experimental groups in the time of escape latency to find the masked platform ($P > 0.05$). In contrast, during the last training day, the findings revealed a highly significant difference in the escape latency time between diabetic control ($P < 0.001$), stressed diabetic group (no treated) ($P < 0.001$), and normal control group. Stressed diabetic group (no treated) displayed remarkably higher escape latency from the 2nd training day to 5th day compared to other groups. On the 5th training day, Two-way ANOVA showed that chronic treatment with insulin only or combined with PFC significantly decreased the escape latency time in chronically stressed diabetic rats ($P < 0.01$, $P < 0.001$), respectively, compared to the stressed diabetic rats without treatment. The combination treatment had no significant influence ($P > 0.05$) on escape latency time compared to diabetic rats treated only with insulin.

Furthermore, in the MWM test the reference memory capacity was assessed by evaluating the time spent in

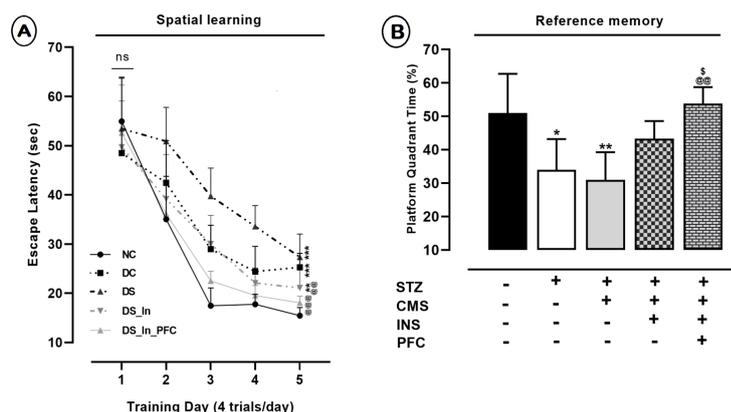


Figure 6. Effects of chronic treatment of insulin combined with phenolic fraction concentrate (12 weeks) on spatial learning (A) and reference memory (B) in normal and streptozotocin (STZ)-induced diabetic rats exposed to chronic mild stress (CMS). Results are presented as Mean \pm SD (N=8). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. normal control group. @@ $P < 0.01$, @@@ $P < 0.001$ vs. untreated STZ-induced diabetic groups exposed to CMS. § $P < 0.05$ vs. STZ-induced diabetic group exposed to CMS and treated only with insulin (two-way repeat measure ANOVA followed by Tukey's multiple comparison test). NC: Normal control. DC: Diabetic control. DS: Diabetic stressed. DS_In: Diabetic stressed treated with Insulin. DS_In_PFC: Diabetic stressed treated with insulin combined with PFCs.

the virtual platform quadrant (Figure 6B). During the probe trial session (90 second), diabetic control rats and diabetic rats exposed to CMS spent significantly the lowest time in the virtual platform quadrant ($P < 0.05$; $P < 0.01$) respectively, compared to the normal control rats. The insulin treatment had no significant influence ($P > 0.05$) on the platform quadrant time compared to the control stressed diabetic rats ($P > 0.05$). The combination treatment revealed a significant effect ($P < 0.01$) on the platform quadrant time compared to the control stressed diabetic rats. In fact, the chronically stressed diabetic rats treated with insulin combined with phenolic fractions concentrate for 12 weeks performed significantly better in the virtual platform quadrant compared to the diabetic rats treated only with insulin ($P < 0.05$).

Effects of insulin combined with phenolic fractions concentrate on tumor necrosis factor-alpha in chronically stressed diabetic rats

The TNF- α level in the brain areas (prefrontal cortex, hippocampus, and striatum) and blood serum of normal rats, diabetic rats, and chronically stressed diabetic rats were evaluated (Figure 7A, B). Across the twelve weeks of the treatment period, TNF- α level in the prefrontal cortex, hippocampus, and striatum increased significantly ($P < 0.05$) in control diabetic rats and chronically stressed diabetic groups compared to the normal control group (Figure 7A). The same results were observed in the blood serum of these groups (Figure 7B). Likewise, stressed diabetic group treated with insulin exhibited a significant increase in TNF- α levels in the brain areas, as well as in the blood serum, compared to the control group. TNF- α levels in insulin-treated stressed diabetic rats significantly decreased in the prefrontal cortex ($P < 0.05$), hippocampus ($P < 0.001$), striatum ($P < 0.001$), and blood

serum ($P < 0.001$) compared to the dramatically increase observed in stressed diabetic rats without treatment. The combination treatment significantly protected stressed diabetic rats against TNF- α elevation induced by diabetes and stress in all the brain areas, as well as in the blood serum. This combination possessed significant effect, stabilizing TNF- α to its normal levels in the hippocampus ($P < 0.01$) and the blood serum ($P < 0.05$) when compared to diabetic rats treated only with insulin.

Discussion

Chronic hyperglycemia-induced inflammation in various organs, especially in the central nervous system (CNS), can increase neuropathy symptoms, which might explain cognitive impairments in diabetics (37). T1DM is often a prime cause of stress (9). Consequently, chronic stress deteriorates cognitive functions in the diabetic rat model (19). The primary goal of this study was to investigate the neuroprotective effect of chronic insulin treatment combined with PFC on learning and memory performances as well as TNF- α level in the prefrontal cortex, hippocampus, striatum, and in the blood in diabetic rat model exposed to CMS.

In the current study, exposure to T1DM and CMS induced a remarkable elevation in FBG associated with a significant increase in corticosterone levels. The obtained results corroborate other research findings conducted in STZ-induced diabetic rats exposed to chronic restraint stress, showing a significant increase in FBG and serum corticosterone levels (16). Also, a previous study revealed that stress impacted blood glucose and systemic insulin resistance in STZ-induced diabetic rats (19). It has been established that stress stimulates the suprarenal gland cortex to secrete corticosterone. This stimulation might be linked with altered activity of the HPA axis (38,39).

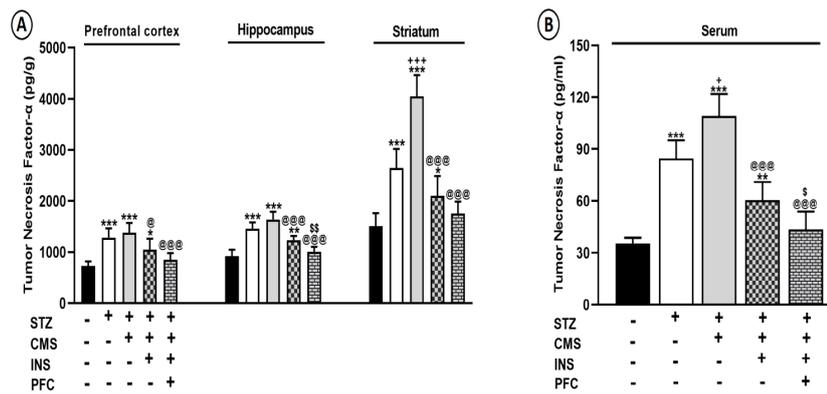


Figure 7. Effects of chronic treatment of insulin combined with phenolic fraction concentrate (12 weeks) on Tumor Necrosis Factor-alpha level in brain homogenates structures (A) and serum (B) in normal and streptozotocin (STZ)-induced diabetic rats exposed to chronic mild stress (CMS). Results are expressed as mean \pm SD (N=6-7). * P < 0.05, ** P < 0.01, *** P < 0.001 vs. normal control group. + P < 0.05, +++ P < 0.05 vs. untreated STZ-induced diabetic group. @ P < 0.05, @@ P < 0.01, @@@ P < 0.001 vs. untreated STZ-induced diabetic groups exposed to CMS. \$ P < 0.05, \$\$ P < 0.01 vs. STZ-induced diabetic group exposed to CMS and treated only with insulin (one-way ANOVA followed by Tukey's multiple comparison test).

Therefore, the abnormal elevation in the production of corticosterone during stress situations appears to be a determinant for the defense system to be put into place. Changes in stress hormones can affect glucose homeostasis in diabetic patients (11). Previous work suggests that high concentrations of corticosterone could be deleterious to beta cells that produce insulin (12). In this study, chronic treatment with insulin combined with PFCs for 12 weeks significantly reversed these variations. This combination has been revealed to possess significant effects, stabilizing glycemia and corticosterone to normal levels when compared to diabetic rats treated only with insulin. A recent study has shown that monotherapy treatment with insulin did not protect patients against cognitive dysfunction and emotional disorders, including stress-related to T1DM (40). The exhibited PFCs effects might be explained by their significant antioxidant and antidiabetic effects (25). As a result, previous research discovered that some phenolic acids such as caffeic and gallic acids protected rodents against chronic stress-induced disturbances (41,42). We suggest that the observed effects of PFCs in diabetic rats exposed to CMS might be linked to their significant amounts of phenolic acids, especially caffeic acid (25). In this context, interest in phenolic acid compounds has increased, mainly due to their anti-inflammatory, antioxidant, and antidiabetic properties against numerous chronic diseases.

To our knowledge, there are no studies exploring the modeling effects of insulin supplemented with PFCs as a rich source of antioxidant compounds on the immune system in diabetic rats exposed to CMS. In the present study, diabetic rats exposed to CMS for 12 weeks exhibited an increase in the prefrontal cortex, hippocampus, and striatum levels of TNF- α . In a disease state, however, the resulting unsuitable neuro-inflammation causes negative actions that far outweigh the normal effects, promoting other disturbances (43). A previous study discovered

that periphery administration of TNF- α influences brain functions by inducing neuro-inflammatory responses (44). Hence, strategies to reduce the overproduction of inflammatory cytokines might have significant neuroprotective effects on diabetes (21). Treatment of stressed diabetic rats in the current study with insulin supplemented with PFCs markedly decreased the brain area levels of TNF- α . More than that, this combination possessed a significant effect by stabilizing TNF- α to its normal levels in the hippocampus and in the blood when compared to diabetic rats treated only with insulin. Furthermore, it has been proved that date seeds extract can act as an anti-inflammatory agent due to its ability to control inflammatory mediators, viz. IL-1, IL-6, IL-2, and TNF- α (26) and suppress the NF- κ B translocation. Furthermore, another study conducted by Saryono et al (27) revealed that the expression of COX-1, COX-2, TGF- β , and IL-1 β in adult women was remarkably reduced after consuming 2.5 g of date seeds/day for 14 days.

Neuroinflammation can damage cognitive functions with relevance to dementia (45). The findings of the present study revealed that both diabetes and stress significantly impair STM and LTM memory, spatial working memory, spatial long-term memory, spatial learning, and reference memory in the diabetic rats exposed to CMS for 12 weeks. In this context, an experimental study revealed that diabetic mice exhibit increased pro-inflammatory cytokine levels, including TNF- α , which is significantly associated with impaired spatial-recognition memory (46). It has been previously established that upregulation of TNF- α inhibits insulin signaling pathways leading to cognitive decline (47,48). It appears that being exposed to diabetes and stress can induce pathophysiologic variations in the CNS. Also, these variations can be manifested as spatial learning and reference memory performance dysfunctions (19). Several studies have revealed that chronic stress could negatively impact the CNS and consequently damage memory

processing (49). Stress altered various mechanisms, including reductions of neurogenesis, synaptic plasticity, neuronal death, and loss of dendritic branches (50). It has been shown that the activation of the HPA axis caused by chronic stress results in adrenal hyperplasia and high glucocorticoid production, which can impair the neural circuits in the regions that are implicated in learning and memory processes, especially the hippocampus (51). These damages are mainly due to the overproduction of TNF- α in the rat hippocampus (52). Our findings revealed that diabetes associated with stress significantly increased TNF- α levels in rat hippocampus. Inflammation can be a crucial reason for neuronal damage in the hippocampus for stressed diabetic rats, which could explain the observed learning and memory impairment in this rat model.

Cumulative evidence has revealed that insulin plays a crucial role in learning and memory performances via modeling several processes such as synaptic plasticity, metabolic control, and neuronal growth regulation. However, interruption of insulin secretion induces deficits in associative learning and memory formation (53). In the hippocampus, the insulin and its receptors influence learning and memory functions through actions on different signaling pathways. The insulin receptor seems to mediate storage and long-term memory processes by activating the signaling Ras/mitogen-activated protein kinase cascade (53). However, the combination treatment with insulin supplemented with PFCs used in this study protected better than insulin the stressed diabetic rats against learning and memory impairment induced by diabetes and stress. This finding revealed that PFCs may decrease brain cell injury in diabetic rats exposed to stress through the attenuation of inflammation, particularly in the hippocampus. This significant effect might be associated with the elevated amount of its phenolic acid compounds, such as chlorogenic acid (584.1 mg/kg) and caffeic acid (597.37 mg/kg) (25). A recent experimental study revealed that caffeic acid treatment significantly attenuated β -amyloid peptide-induced brain cell apoptosis and inflammation in the hippocampus, protecting animals against learning and memory impairment (54). Additionally, another experimental study demonstrated that treatment with chlorogenic acid (30 mg/kg) significantly inhibited the overproduction of pro-inflammatory cytokines TNF- α and IL-2, and enhanced the expression of anti-inflammatory cytokines IL-4 and IL-13 in the CA1 region of the hippocampus (55). We strongly suggest that PFCs might be a significant neuroprotective product against learning and memory disturbances induced by diabetes and stress through the regulation of anti-inflammatory and pro-inflammatory cytokines. Moreover, polyphenol supplementation has been reported to exhibit protective effects on brain regions through the ability to protect brain cells against damage, an ability to attenuate neuroinflammation and the potential to promote learning and memory (56).

In conclusion, the present study is the first to enlighten the anti-inflammatory action of the insulin treatment supplemented with PFCs over insulin monotherapy. We demonstrated that this combination significantly attenuated neuroinflammation, especially in the hippocampus of diabetic rats exposed to CMS. This combination treatment markedly protected the rats against learning and memory impairment, potentially mediated by its capacity to protect brain cells against inflammation triggered by stress and diabetes.

Authors' contributions

BS and YA invented the work's conception, performed the experiments and wrote the draft; EM performed the experiments and helped in manuscript preparation; NF and HA 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 animal procedures were performed in accordance with the NIH guidelines published in the "Guide for the Care and Use of Laboratory Animals" published by the National Institute of Health and approved by the Animal Ethics Committee of Nutrition, Food and Health team, Biology and Health laboratory, Faculty of science, Ibn Tofail University, Kenitra, Morocco (NUT/01/93. dated October, 2021).

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