CHRONIC CAFFEINE'S EFFECTS ON BEHAVIOURAL CHANGES IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

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Abstract

Context. Memory deficits, anxiety and depression are often associated with diabetes, worsening diabetic patients' prognosis. Caffeine, a worldwide used psychoactive substance, is a candidate for improving these conditions.

Objective. The aim of this study was to assess the behaviour in streptozotocin-induced diabetic Wistar rats and to evaluate the behavioural effects of caffeine administration.

Materials and methods. Diabetes was induced by a single intraperitoneal injection of 50 mg/kg BW streptozotocin (n=10), while control rats received the vehicle (n=9). After six weeks, behavioural tests for anxiety, memory and depression were performed: elevated plus maze (EPM) test, novel object recognition (NOR) test and forced swimming test (FST), respectively. The tests were repeated after further 2 weeks of continuous caffeine administration (20 mg/kg BW/day in drinking water).

Results. Diabetic rats manifested a high anxiety level, showed by a reduced exploratory activity compared to control rats ($p<0.05$) and long-term memory impairment, spending more time near the old object in NOR test. Caffeine administered for 2 weeks did not modify glycemic values in either group, and attenuated the behavioural changes observed in the EPM test. Also, in NOR test for long-term memory, caffeine administration induced an increased time spent with the novel object than with the old one in both groups.

Conclusions. Our data suggest that chronic caffeine administration has an anxiolytic effect in diabetic rats and improves long-term memory in both diabetic and control rats.

Key words: diabetes, streptozotocin, caffeine, anxiety, depression, memory, rats.

INTRODUCTION

Diabetes mellitus is considered one of the major health care problems of the 21st century, with extreme medical and economic consequences, having a prevalence of one in eleven adults worldwide with a

tendency of further increasing (1). Diabetes mellitus manifests with hyperglycemias due either to lack of insulin secretion, or to defects of insulin action upon its receptors, or both (2). Depression is a co-morbid condition in chronic illness in general, but its prevalence increases two times in type II diabetes and even three times in type I diabetic patients compared to the general population (3). An increase of anxiety in diabetic patients has also been reported (4). As a consequence, depression and anxiety worsen the outcomes of diabetes in these patients, as their quality of life and overall functioning subsequently decrease (5). Even though it is a fact that diabetes leads to structural changes in the brain (6), the association between depression and diabetes remains poorly understood, with still unclear mechanisms that might explain it (7). Recent studies show that the association of depression and diabetes increases the risk of neuropathy (8), nephropathy, retinopathy and macrovascular disease (9). A progressive decline in cognitive function, especially starting with deterioration of memory, is higher in patients with diabetes or altered glucose metabolism (10).

Caffeine is one of the most used self-administered licit psychoactive substances worldwide (11) and it is an unselective A_{2A} and A₁ adenosine receptor antagonist (12). Caffeine intake was proved to decrease anxiety and depression in humans in low doses, while high doses exacerbate anxiety (13). Adenosine seems to be involved in cognitive processes, such as learning and memory: epidemiological studies showed that a regular use of caffeine in humans is associated with less impairment of cognitive function with aging as well as in patients with Alzheimer disease (14). Adenosine and drugs acting on its receptors might represent a new strategy in controlling memory impairment in neuropsychiatric diseases (14). Moreover, coffee consumption is associated with a lower risk of

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diabetes (15) and studies on diabetic rats found that administration of increasing doses of caffeine leads to a decrease in glycemia and to an improved glucose tolerance (16).

As literature studies provide contradictory findings in the relationship between diabetes and behaviour, the purpose of this study was to evaluate the behavioural changes that may occur in streptozotocin-induced diabetic rats and to test the effects of chronic caffeine on behaviour in this animal model.

MATERIALS AND METHODS

Animals

Experiments were performed using 3–4 months old Wistar albino male rats, weighing 300–350 g at the beginning of the experiments. All animals were kept in standard illuminating conditions (12 hours' day cycle), in transparent cages, at constant temperature (22±2°C), with free access to food and water. Animals were provided by "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania. All animal procedures were carried out with the approval of the local ethics committee in accordance with the European Communities Council Directive 2010/63/EU on the protection of animals used for scientific purposes.

Chemicals and Study Design

Diabetes was induced by a single intraperitoneal injection of 50 mg/kg BW streptozotocin (STZ) (Sigma-Aldrich) diluted in 0.9% saline solution. Streptozotocin induces diabetes within three days by destroying the beta cells (17). Aged-matched control rats received an equivalent of 0.9% saline solution. Blood samples from fasting rats were taken from the tail vein after one week of STZ or vehicle injection. Rats with fasting glycemia over 250 mg/dL were considered diabetic and were included in the study. Rats with glycemia over 600 mg/dL were eliminated from the study due to poor biological potential that would not ensure long-term survival (n=3). In the end 19 animals were included: 9 in the control group and 10 in the diabetic group.

Anhydrous caffeine (1,3,7 - trimethylxanthine) (Sigma-Aldrich) – 20 mg/kg BW/day – or water placebo, was given orally for 2 weeks (starting the next day after the last behavioural test in diabetic and control rats was performed) and then continued throughout the experiments to a subgroup of the diabetic (n=5) and control rats (n=5). The amount of caffeine intake for each rat was estimated by measuring daily the water consumption one week before starting caffeine's administration.

Behavioural tests

Six weeks after STZ injection, anxiety-like behaviour of all rats was evaluated with Elevated Plus Maze Test (EPM). Depression like behaviour was evaluated with Porsolt forced swimming test (FST). Short and long-term memory was assessed with Novel Object Recognition Test (NOR). All the behavioural tests were done in the same time intervals, 8:00–12:00 and 14:00–17:00, in order to reduce circadian rhythms effect. Noldus Ethovision XT 4 video tracking system was used to record and analyze automatically the behavioural tests.

After the first behavioural tests, 10 of the rats were administered caffeine orally in the drinking water (5 control rats, 5 diabetic rats). All the behavioural tests were repeated after 2 weeks of caffeine administration as described above in this section.

Elevated Plus Maze Test

The elevated plus maze was conducted as previously described (18, 19). In short, the maze consisted of a black cross-sign shaped area with 50 cm long and 10 cm wide arms placed at 50 cm from the floor. Two arms were opened and two closed with 40 cm height lateral walls. The rat was placed at the crossing of the four arms with the face towards an opened arm and let to explore the maze for 5 minutes. The total distance moved (cm), the time spent in each arm (s), the number of entries in each arm, the speed (cm/s) and the number of defecations were evaluated.

Forced Swimming Test

A modified Porsolt forced swimming test was used (20). One rat at a time was placed in a glass cylinder 45 cm in height and 30 cm in diameter. The water level was about 30 cm in height, so that the rat could not touch the bottom of the cylinder during swimming; the water temperature was 23–26°C. The water was changed every two rats. All animals were task-preconditioned for 5 minutes, 24h before the test. During the test, which lasted for 5 minutes, rat's mobility/immobility time was measured.

Novel Object Recognition Test

This test evaluates the working memory in adult rats, based on the ability of the animal to distinguish novel from familiar stimuli (21). First described by Ennaceur and Delacour (22), the test exists in various forms. Our test was done as previously described (19) and was divided in two parts, each 4 minutes long, separated by a variable interval of 5 minutes – in order to assess short-term memory – and 24 h – in order to assess long-term memory. In the first part of the test rats had to explore and get familiarized with an arena

with black walls (40/40/40 cm) where two objects were left. The objects used in the test had no natural significance for rats. In the second part of the test, the rats were put back in the arena with one of the old objects and a new object and we quantified the time spent exploring each object. The objects were always placed in the same positions. Exploring an object was defined as directing the nose at a distance ≤ 2.5 cm to the object and/or touching it with the nose. The novel object had a different color, shape and texture than the old objects. We used glass material for the old objects and plastic for the new object. Before this test sessions, the rats had been previously familiarized with the arena, as we used the same arena without objects to perform an Open Field Test, whose results had been already reported (23). If the actual working memory of the animal is functional, in the second part of the test, the time spent exploring the new object would be significantly longer, the old object being easily recognized. When presenting a novel stimulus together with a familiar one, the animal explores the novel stimulus more, until this stimulus loses its novelty (24).

Statistical analysis

The data were analyzed using SPSS 22.0. The distribution of the data was checked for normality using the Shapiro-Wilk test and parametrical tests were employed (T-test, repeated-measures, respectively one-way ANOVA, ANCOVA, Pearson's correlation coefficient) for continuous variables with Shapiro-Wilk $p > 0.05$, otherwise, non-parametrical tests (Mann-Whitney-U, Kruskal-Wallis, Wilcoxon's Signed Ranks Test, Spearman's correlation coefficient) were used. In the case of normally distributed values, the results are presented as mean $\pm$ standard error of the mean (SEM). If the data were not normally distributed or not continuous, the results are presented as median value and the inter quartile range (IQR). The IQR was chosen as it gives a very good indicator for the distribution and spread of the data. As, for non-parametric analysis, we are comparing mean ranks, it is very important to know this value, for the analysis of the compared variances between the groups. The statistical significance is reported for the two-tailed test, unless otherwise mentioned. The glycemic values are reported as mean $\pm$ SEM. P-values were highlighted in the graphs only where statistical significance was obtained.

RESULTS

Effects of STZ and caffeine on plasma glucose level

The plasma glucose level raised in all diabetic rats after STZ injection, with a glycemic value of 455.50 ± 90.30 mg/dL after one week from the induction of diabetes and 517.40 ± 76.96 mg/dL after 6 weeks ($p < 0.01$). The glycemia of control rats did not differ across the study (113.60 ± 8.77 mg/dL after one week from vehicle injection, 102 ± 5.63 mg/dL after 6 weeks).

Caffeine administration did not modify glycemic values either in the control group, or in the diabetic one (the glycemia in diabetic rats was 523.30 ± 75.71 mg/dL and the glycemia in control rats was 99.60 ± 7.65 mg/dL after 2 weeks of caffeine administration).

Behavioural tests in diabetic rats

Regarding the EPM test, the rats in the control group had significantly more entries in both the opened arms in the control group, the median number of opened arms entries=3, IQR=5.5, and in the diabetic group the median number of opened arms entries =1, IQR=3, $Z=65.5$, $n_1=10$, $n_2=9$, $p=0.047$ (one-sided)] and in the closed arms (in the control group, the median number of closed arms entries=6, IQR=20, in the diabetic group the median number of closed arms entries=2, IQR=4.25, $Z=72.5$, $n_1=10$, $n_2=9$, $p=0.022$). Moreover, there were differences between the mobility time of the rats in the two groups ($p=0.022$, $Z=73$, median control group mobility time=108.32 s, IQR=103.36, median diabetic group mobility time=52.96 s, IQR=24.82). The differences in both closed and opened arms entries remained after adjusting for the mobility status (ANCOVA analysis closed arms entries $F(1)=21.214$, $p < 0.001$, ANCOVA analysis opened arms entries $F(1)=9.407$, $p=0.007$). There were no significant differences in the time spent in the opened arms adjusted to the mobility status (ANCOVA analysis $F(1)=0.127$, $p=0.726$, 95%CI=[1.107,14.890] in the diabetic group, respectively 95%CI=[2.497,17.150] in the control group).

The FST showed no significant differences between the two groups regarding the time of mobility.

The NOR test revealed no differences between the diabetic and control group for short-term memory. However, at long-term memory test, the rats in the control group spent more time at the novel object than the diabetic rats (median time diabetic group=1.4 s, IQR=4.42, median time control group=76

Table 1. Results in elevated plus maze test: the median and IQR (inter-quartile-range) for the closed arms entries, opened arms entries and defecations before and after caffeine administration in each group

Group	Elevated plus maze test											
	Before caffeine administration						After caffeine administration					
	Closed arms entries	Opened arms entries	Defecations	Closed arms entries	Opened arms entries	Defecations	Closed arms entries	Opened arms entries	Defecations	Closed arms entries	Opened arms entries	Defecations
	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR
Diabetes	2	1.5	1	2.5	3	3.5	10	16	3	2	0	2.5
Control	5	4	2	4	0	2	19	27.5	5	3.5	0	1

s, IQR=13.76, $Z=71$, $n_1=10$, $n_2=9$, $p=0.032$). In the diabetic group, the time spent at the novel object was shorter than the time spent at the old object (median time novel object=1.4 s, IQR=4.42, median time old object=10.32 s, IQR=41.28, $Z=43$, $p=0.015$).

Behavioural tests in caffeine treated diabetic rats

Regarding the EPM test, the number of entries in the closed arms increased after caffeine administration in the diabetic rats [$Z=-1.753$, $p=0.04$ (one-tailed)]. The control rats still had more entries in the opened arms after caffeine intake than the diabetic rats [$Z=-1.702$, $p=0.047$ (one-tailed)]. The number of defecations decreased for the diabetic rats after caffeine administration ($Z=-2.041$, $p=0.041$) (results detailed in Table 1).

Figure 1. Box-plot of the mobility time at forced swimming test (FST). The mobility of diabetic rats decreased significantly after caffeine administration [$Z=-1.753$, $p=0.04$ (one-tailed), median mobility time for diabetic rats before caffeine=210 s, IQR=77.5, median mobility time for diabetic rats after caffeine=185 s, IQR=45]. The rats in the control group had similar mobility time after caffeine administration as before it, however, they were significantly more mobile than diabetic rats after caffeine ($Z=-2.402$, $p=0.016$, median mobility time for diabetic rats=185 s, IQR=45, median mobility time for control rats=265 s, IQR=62.5). The bullets represent significant outliers. DBC - diabetic rats before caffeine administration, DAC - diabetic rats after caffeine administration, CBC - control rats before caffeine administration, CAC - control rats after caffeine administration.

In FST, the mobility of diabetic rats decreased significantly after caffeine administration. The rats in the control group had similar mobility time after caffeine administration as before it, however, they were significantly more mobile than diabetic rats after caffeine (Fig. 1).

At the novel object recognition test, the rats in the diabetic group, spent more time with the novel object than with the old one, at long-term memory testing after caffeine administration ($Z=-1.483$, $p=0.049$, time spent with novel object after caffeine administration=9.68 s, IQR=10.52, time spent with old object after caffeine administration=5.84 s, IQR=7.24) (Fig. 2 A). Conversely, at the short-term memory testing, diabetic rats spent less time with the novel object after caffeine administration ($Z=-2.023$, $p=0.043$, time spent with novel object before caffeine administration=8.4 s, IQR=67.68, time spent with novel object after caffeine administration=2 s, IQR=6.92) (Fig. 2 B). After caffeine administration, the rats in the control group, only spent more time at the novel object at the long-term memory testing ($Z=-2.023$, $p=0.043$, time spent with novel object before caffeine administration=3.52s, IQR=7.56, time spent with novel object after caffeine administration=14.16 s, IQR=20.36) (Fig. 2 A). After caffeine administration, the rats in the control group spent more time with the novel object than with the old one at long-term memory testing ($Z=-2.023$, $p=0.043$, time spent with novel object after caffeine administration=14.16s, IQR=20.36, time spent with old object after caffeine administration =3.36s, IQR=3.64)(Fig. 2 A).

DISCUSSION

The main finding of the present study is that chronic administration of low to moderate doses of caffeine improves long-term memory in both diabetic and control rats. Chronic hyperglycemias after the streptozotocin injection in rats alter hippocampal metabolism (25), with a consecutive increase in adenosine A2 receptors in the hippocampus (26). The blockage of these receptors by caffeine may explain

Figure 2. Novel object recognition (NOR) test for short-term and long-term memory assessment. **A.** Bar-plot of the time spent near the novel and old object at the NOR for long-term memory test for each group, before and after caffeine administration. **B.** Bar-plot of the time spent near the novel and old object at the NOR for short-term memory test for each group, before and after caffeine administration. The error bars represent the standard error of the mean. DBC—diabetic rats before caffeine administration, DAC - diabetic rats after caffeine administration, CBC- control rats before caffeine administration, CAC- control rats after caffeine administration.

its memory benefits and the potential of preventing hippocampal degeneration. Other studies have proven a protective effect of caffeine when it was administered prior to diabetes induction (27), by decreasing blood glucose concentration (28) and protecting pancreatic beta cells against streptozotocin-induced damage (29). For the current experimental design, caffeine was administered after diabetes induction and did not modify the glycemic state, but improved the results at the NOR test for long-term memory.

Long-term administration of caffeine was found to increase hippocampal dopamine and serotonin levels in rats (30), so caffeine could play a role as an antidepressant agent and might facilitate long-term memory consolidation. The FST is one of the most used behavioural tests for screening antidepressant drugs. When rats are forced to swim in escapeless conditions, after an initial period of struggling, they adopt an immobile posture that reflects a lower mood. As antidepressant drugs reduce the immobility time of rats, this behavioural despair condition is considered similar to a depression-like state (20). In the present study, diabetic rats had similar immobility times compared with control rats after 6 weeks of high glycemic levels. Some studies found significant differences in immobility time in streptozotocin-induced diabetic rats after 21 days (31) or 28 days

from streptozotocin injection (32, 33), whereas others reported no differences after 21 days, but only after 28 days from streptozotocin injection (33). We may assume that the heterogeneity of these findings could result from the large variability of the study-designs, including different doses of streptozotocin used for diabetes induction (31), or the specific features of the animals subjected to experiments (like weight or age) (33). However, in our study, after 2 weeks of caffeine administration, diabetic rats were less mobile at FST than before caffeine administration, while mobility time in the control group remained unchanged. This depressive behaviour of diabetic rats at 8 weeks after streptozotocin injection could be a consequence of a longer duration of diabetic pathology.

The mobility at EPM was higher in the control group, which is in accordance with other studies that found an impaired locomotor activity in diabetic rats (34). Caffeine administration had a negative impact in the control group, decreasing their exploratory activity, but this effect was not observed in diabetic rats. Our findings are supported by other recent data. Caffeine administration has been proven to decrease the total distance moved when control Wistar rats were subjected to a locomotor test, and showed a decrease of closed arms entries when exploratory activity of the rats was assessed by EPM testing (35).

Therefore, caffeine seems to have an anxiogenic effect in non-diabetic Wistar rats. Marin *et al.* also found a locomotor stimulation after low acute doses of caffeine administration, but a locomotor depression after high acute doses of caffeine in adult Wistar rats (36). Even though we have not used high caffeine doses, we have employed a chronic model of administering low to moderate doses of caffeine. Previous studies have suggested that low doses of caffeine have a stimulant effect mediated by A2A receptor blockade, while the depressant effect might be explained by A1 receptor blockade (37).

The control rats had more entries in both the opened and closed arms of EPM than the diabetic rats, but adjusted to the mobility status, the time spent in the opened arms was the same. The diabetic rats had less exploring capacity and we might correlate it with an increased level of anxiety. Chronic caffeine administration increased the number of closed arms entries and decreased the number of defecations for the diabetic rats. We consider these effects to account for a small reduction in anxiety. However, the effects of caffeine on anxiety are still disputed. While some studies have indicated an anxiolytic influence (38, 39), others have observed an anxiogenic effect induced by caffeine administration (40). We found an anxiogenic effect in the control group, but an anxiolytic effect of caffeine in diabetic rats. Considering that higher concentrations of caffeine increase anxiety, while lower doses decrease it in humans (41), we suggest that caffeine may interact with different receptors involved in behavioural control depending on whether the rat has diabetes or not, as diabetes might interfere with the expression of different types and numbers of receptors (26).

It should be mentioned that type I diabetes has the overall sex ratio equal in children diagnosed under the age of 15, and, in the European populations, an approximate 3:2 male: female ratio (42). So, type I diabetes is a disorder without a strong female bias. Also, it has been previously reported that caffeine administration interferes with gonadotropic hormones secretion and with plasma progesterone concentrations in both male and female rats (43). Moreover, it has been reported that caffeine administration highly increases locomotion in females (44). However, the specific mechanisms involved in these changes and their overall dose-dependent behavioural effects in females are yet under research. In order to minimize the potential behavioural differences induced by the interactions between the caffeine administration and

the estrous cycle, only male rats were used in these experiments.

The more time passed since diabetes was induced in rats, more behavioural changes manifested: anxiety, memory impairments or depression. These behavioural changes may be due to changes in brain metabolism, affecting serotonin and adenosine systems. Caffeine administration decreases locomotor activity in control rats, but not in diabetic ones; it has an anxiolytic effect in diabetic rats, and it improves long-term memory in both diabetic and control rats.

In conclusion, this study suggests a beneficial effect on behaviour induced by chronic low-moderate doses of caffeine in diabetic rats. Further studies are needed in order to reveal the possible involved mechanisms. Given the wide availability of caffeine, our results could be translated into type I diabetes research in humans.

Conflict of interest

The authors declare that they have no conflict of interest concerning this article.

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