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Effects of chlorogenic acid, caffeine and coffee on components of the purinergic system of streptozotocin-induced diabetic rats☆

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Abstract

We evaluated the effect of chlorogenic acid (CGA), caffeine (CA) and coffee (CF) on components of the purinergic system from the cerebral cortex and platelets of streptozotocin-induced diabetic rats. Animals were divided into eight groups: control animals treated with (I) water (WT), (II) CGA (5 mg/kg), (III) CA (15 mg/kg) and (IV) CF (0.5 g/kg), and diabetic animals treated with (V) WT, (VI) CGA (5 mg/kg), (VII) CA (15 mg/kg) and (VIII) CF (0.5 g/kg). Our results showed an increase (173%) in adenosine monophosphate (AMP) hydrolysis in the cerebral cortex of diabetic rats. In addition, CF treatment increased adenosine diphosphate (ADP) and AMP hydrolysis in group VIII synaptosomes. Platelets showed an increase in ectonucleotidase activity in group V, and all treatments reduced the increase in adenosine triphosphate and ADP hydrolysis. Furthermore, there was an increase in platelet aggregation of 72% in the diabetic rats, and CGA and CF treatment reduced platelet aggregation by nearly 60% when compared to diabetic rats. In this context, we can suggest that CGA and CF treatment should be considered a therapeutic and scientific target to be investigated in diseases associated with hyperglycemia.

Keywords: Caffeine; Chlorogenic acid; Coffee; Diabetes mellitus; Purinergic system

1. Introduction

Data demonstrate that, in 2012, around 1.5 million deaths were directly caused by diabetes mellitus (DM). In 2014, the global prevalence of diabetes was estimated to be 9% among adults more than 18 years old. The World Health Organization predicts that diabetes will be the seventh leading cause of death in 2030 [1]. DM is a condition characterized by hyperglycemia resulting from the body's inability to use blood glucose adequately. In type 1 diabetes, the pancreas does not release enough insulin, and therefore, intake of blood glucose into cells cannot be stimulated. Type 2 diabetes is characterized by decreased pancreatic synthesis and release of insulin combined with insulin resistance [2].

Chronic hyperglycemia increases the frequency of nonenzymatic glucose adduction with proteins, lipids and DNA to form advanced glycation end-products (AGEs), which alter the structure and function of involved proteins. AGEs have been associated with chronic complications of DM and aging-related diseases, such as nephropathy, chronic renal insufficiency, Alzheimer's disease, neuropathy and cataracts [3].

Adenine nucleotides and nucleosides have well-known cell signaling functions. Adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine have important actuation in neurotransmission [4] and neuroprotection in the central nervous system (CNS) [5], as well as acting in vascular tone and thromboregulation [6,7]. The regulation of those simple molecules can be important for the prevention of pathology related to platelets and neurons [8,9].

Enzymes that degrade adenine nucleotides NTPDases and 5′-nucleotidases regulate the molecules' concentration. Robson and collaborators showed that NTPDases have acute effects on cellular metabolism, adhesion, activation and migration, inclusive of cellular proliferation, differentiation and apoptosis [10]. 5′-Nucleotidase functions are related to inactivation and catabolism of ATP and adenosine formation [11].

These enzymes can be altered in hypertension, hypercholesterolemia and cancer [12], as well as in hyperglycemia conditions [13]. Research with diabetes has demonstrated that these enzymes are found in increased levels in diabetic patients and experimental diabetes models [9,13,14]. On the other hand, fruits and vegetables rich in polyphenols provide vascular protection, which may be useful

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in the prevention and progression of the pathologies cited above. Furthermore, red wine, grape juice and some of their polyphenols have been shown to prevent these alterations [15].

Studies have shown that consumption of coffee has been associated with a lower risk of development of DM. This beneficial effect of coffee on diabetic status has been attributed mainly to the properties of caffeine and chlorogenic acid [16]. Chlorogenic acid is a polyphenol present in many plants [17]. Studies have established that chlorogenic acid has a range of properties such as antioxidant [18], anti-inflammatory [19], neuroprotective [20] and hypoglycemic [21]. On the other hand, caffeine is a methylxanthine, nonselective antagonist of adenosine receptors (A_1 and A_{2A}), and its effects have been related to glucose metabolism through alteration of glucose uptake, modulating glycogen metabolism and reduction of carbohydrate storage [22].

Our research group has demonstrated that coffee extracts and coffee compounds (chlorogenic acid and caffeine) protected the CNS from hyperglycemia-induced alterations [23]. In addition, platelets can suffer an increase in reactivity due to high glucose levels, and natural compounds such as caffeic acid reduce platelet activity [24]. Although studies with the cerebral cortex and platelets are described, research involving these compounds and ectonucleotidases in both tissues has not been described. In line with these findings, we investigated whether chlorogenic acid, caffeine and coffee could protect the alterations of key enzymes that regulate purinergic neurotransmission in the brain, and platelet aggregation of streptozotocin (STZ)-induced diabetes.

2. Experimental procedure

2.1. Chemicals

ATP, ADP, adenosine monophosphate (AMP), adenosine, Percoll, Tris buffer (tris(hydroxymethyl)aminomethane) GR, Coomassie brilliant blue G, HEPES, STZ (SID 24899428), chlorogenic acid (CID 1794427) and caffeine (CID 2519) (approximately 99% purity) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used in the experiments were of analytical grade and of the highest purity. Coffee used in the experiment was soluble, lyophilized and 100% from *Coffee arabica* species, analyzed by high-performance liquid chromatography (HPLC) by Passamonti and colleagues (Department of Life Sciences, University of Trieste, Italy). The dose of caffeine was found to be 30 mg of caffeine per gram of coffee, and we chose 15 mg/kg, a dose corresponding to an intake of approximately three cups of coffee in humans [25]. The chosen dose of chlorogenic acid was determined in accordance with literature data [26].

2.2. Animals

Adult male Wistar rats (70–90 days old; 220–300 g) from the Central Animal House of the University Federal of Santa Maria (UFSM) were used in this research. Animals were maintained at 23°C±1°C on a 12-h light/dark cycle with free access to food and water. All animal procedures were approved by the UFSM Animal Ethics Committee (protocol under number: 23/2011).

2.3. Experimental induction of diabetes

Diabetes was induced by a single intraperitoneal injection of 60 mg/kg STZ diluted in 0.1 M sodium citrate buffer (pH 4.5) [27]. Control rats received the vehicle (sodium citrate buffer). To avoid hypoglycemic shock, treated rats received 5% glucose for 24 h after STZ induction. After 7 days, a small amount of blood was taken from the tail vein to determine glucose levels, which were measured with a portable glucometer (ADVANTAGE; Boehringer Mannheim, MO, USA). Rats with glycemia over 250 mg/dl were chosen to study. Subsequently, the measurement of blood glucose and treatment of animals with the compounds was begun.

2.4. Treatment with chlorogenic acid, caffeine and coffee

Rats were divided into eight groups (n=10): control animals treated with (I) water (WT), (II) chlorogenic acid (CGA 5 mg/kg), (III) caffeine (CA 15 mg/kg) or (IV) coffee (CF 0.5 g/kg), and diabetic animals treated with (V) WT, (VI) CGA (5 mg/kg), (VII) CA (15 mg/kg) or (VIII) CF (0.5 g/kg). Rats in control groups received water. Chlorogenic acid and caffeine were freshly prepared in water. Coffee was solubilized in hot water. The solutions were administered *via* gavage once a day for 30 days.

2.5. Preparation of samples and enzyme assays

2.5.1. Serum

Total blood was collected and centrifuged at 1800×*g* for 15 min, the precipitate was discarded, and the serum was used to determine fructosamine and glucose levels.

2.5.2. Fructosamine and glucose levels

The glucose and fructosamine levels were determined using commercial kits (Labtest, Minas Gerais, Brazil). Glucose oxidase catalyzes the oxidation of glucose to gluconic acid and hydrogen peroxide. Through an oxidative coupling reaction catalyzed by peroxidase, the hydrogen peroxide formed reacts with 4-aminoantipyrine and phenol to form a red-colored complex (quinone imine); the absorbance was measured at 505 nm and is directly proportional to the glucose concentration in the sample.

Glucose binds to amino groups of proteins forming a Schiff base, which after a molecular rearrangement becomes a stable ketamine called fructosamine. In alkaline pH, fructosamine is converted to its enol form, which reduces nitrotetrazolium blue to a purple compound. The difference in absorbance after 15 min is proportional to the concentration of fructosamine in the sample.

2.6. Brain tissue preparation

The cerebral cortex was collected for nucleotide quantification, NTPDase1, 2 and 3 and 5′-nucleotidase expression in the cerebral cortex. NTPDase and 5′-nucleotidase activities were analyzed in the synaptosomes of the cerebral cortex.

2.6.1. Nucleotide quantification

The denaturation of sample proteins was performed using 0.6 mol/L perchloric acid. All samples were then centrifuged (14,000×*g* for 10 min), and the supernatants were neutralized with 4.0 N KOH and clarified with a second centrifugation (14,000×*g* for 15 min) [28]. HPLC was performed with Shimadzu (Kyoto, Japan) equipment composed of a model LC-20AT reciprocating pump, a model DGU-20A5 degasser, a model SPD-M20A diode array detector (DAD), an autosampler (SIL-20 A) and a model CBM-20A integrator operated by LC Solution 1.22 SP1 software. Purines in the samples (ATP, ADP, AMP and adenosine) were identified by their retention time and DAD spectrum (in the range 200–400 nm) and quantified by comparison of the peak area with standards. The results are expressed as pmol of the different compounds per g of tissue.

2.6.2. Synaptosome preparation

Firstly, the brain was dissected into cerebral cortex, which was homogenized in Medium I buffer pH 7.5. The synaptosomes from the cerebral cortex were prepared as described [29] using a discontinuous

Percoll gradient. Synaptosomes were prepared fresh daily, maintained at 4 C throughout the procedure and used for enzymatic assays.

2.6.3. Determination of ectonucleotidase activity

The activity of NTPDase and 5′-nucleotidase in synaptosomes from the cerebral cortex was determined in accordance with a previous report [30], and 5′-nucleotidase was determined by a previously reported method [31]. The release of inorganic phosphate (Pi) was measured by the method previously described [32]. All samples were carried out in triplicate, and enzyme activity was reported as nmol Pi released/min/mg protein.

2.6.4. Enzyme gene expression

Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with manufacturer's instructions. Total RNA was quantified by spectrophotometry, and cDNA was synthesized with an ImProm-II Reverse Transcription System (Promega) from 1 μg of total RNA. Quantitative polymerase chain reaction (PCR) was performed using SYBR Green I (Invitrogen) to detect double-strand cDNA synthesis. Reactions were done in a volume of 25 μl using 12.5 μl of diluted cDNA (1:50 for *Hprt1*, *Rlp13A*, *entpd1*, *entpd2* and *cd73*), containing a final concentration of 0.5 M betaine (Sigma-Aldrich), 0.2× SYBR Green I (Invitrogen), 100 μM dNTP, 1× PCR buffer, 3 mM $MgCl_2$, 0.25 U Platinum Taq DNA Polymerase (Invitrogen) and 200 nM each of reverse and forward primers (Table 1). The PCR cycling conditions were as follows: an initial polymerase activation step of 5 min at 95°C, 40 cycles of 15 s at 95°C for denaturation, 35 s at 60°C for annealing and 15 s at 72°C for elongation. At the end of the cycling protocol, a melting-curve analysis was included and fluorescence was measured from 60°C to 99°C. Relative expression levels were determined with 7500 Fast Real-Time System Sequence Detection Software v.2.0.5 (Applied Biosystems). The efficiency per sample was calculated using LinRegPCR 11.0 Software (http://LinRegPCR.nl), and the stability of the references genes, *Hprt1* and *Rlp13A* (*M-value*), and the optimal number of reference genes according to the pairwise variation (*V*) were analyzed by GeNorm 3.5 Software (http://medgen.ugent.be/genorm/). Relative RNA expression levels were determined using the $2^{-\Delta\Delta CT}$ method.

2.7. Platelet preparation

Platelet-rich plasma (PRP) was prepared by the method of Lunkes et al. (2004) [33] with the following minor modifications. Total blood was collected by cardiac puncture with 0.120 M sodium citrate as anticoagulant. The blood-citrate system was centrifuged at 1000 rpm for 15 min to produce PRP (used for platelet aggregation). PRP was centrifuged at 3500 rpm for 25 min and washed twice with 3.5 mM HEPES buffer, pH 7.0, containing 142 mM KCl and 5.5 mM glucose. The platelet pellets were suspended in HEPES buffer and used to determine enzymatic activity.

Table 1
PCR primers design

Enzymes	Primer sequences (5′-3′)	GenBank accession number (mRNA)	Amplicon size
Hprt1[a]	F: GCAGACTTTGCTTTCCTTGG R: CGAGAGGTCCTTTTCACCAG	NM_012583	81
Rpl13A[a]	F: ACAAGAAAAAGCGGATGGTG R: TTCCGGTAATGGATCTTTGC	NM_173340	167
entpd1[b]	F: TGAGTGAATACTGTTTCTCGGGGACC R: GCCCAAAGTCCACCCTGCGTTG	NM_022587	134
entpd2[b]	F: CCTTCAATGGCAGTGCCATCGTC R: CTATGAAGTTTCCAGTCACGGGAGG	NM_172030	156
entpd3[b]	F: GGATACAAATTCACTGAGGCAACTTGG R: TGTAGCCTAGGGACCAGGCGATG	NM_178106	88
cd73[b]	F: GGCCAGTCCACAGGGGAGTTCC R: TTGACCAGATAGCTTGGGAGGACC	NM_021576	

[a] According to Bonefeld (2008).
[b] Designed by authors.

2.7.1. Platelet aggregation

PRP described above was used for aggregation tests. The platelet aggregation profile was evaluated by measuring turbidity with a Chrono-log optical aggregometer (AGGRO/LINK Model 810-CA software for Windows v.5.1) using 5 and 10 μmol/L of ADP as agonist [34]. The results were shown as percentage aggregation.

2.7.2. NTPDase and 5′-nucleotidase activity determination

The NTPDase and 5′-nucleotidase enzymatic assay was carried out as previously described [33]. Released Pi was assayed by a method using malachite green as the colorimetric reagent and KH_2PO_4 as standard [32]. All samples were run in triplicate. Enzyme-specific activities are reported as nmol Pi released/min/mg of protein.

2.8. In vitro tests

To evaluate the effect of chlorogenic acid, caffeine and chlorogenic acid plus caffeine in nucleotide hydrolysis in synaptosomes and platelets, *in vitro* experiments were performed using different concentrations of chlorogenic acid, caffeine and chlorogenic acid plus caffeine (at 25, 50, 100, 150 and 200 μM) based on Schmatz et al. (2013) [15]. The chlorogenic acid was diluted in 25% ethanol, and caffeine was diluted in water. They were incubated in the presence of ATP, ADP and AMP as substrates with the incubation medium as described above.

2.9. Protein determination

Protein content was determined colorimetrically using bovine serum albumin as standard [35].

2.10. Statistical analysis

For the *in vivo* results, two-way analysis of variance (ANOVA) statistical analysis was used to assess the interaction between the factors of the test, and it is represented as follows: two-way ANOVA statistical analysis showed a significant control or diabetic×water or treatment (CGA, CA or CF) interaction for random variable [F(DFn,DFd) = value; P = value]. One-way ANOVA followed by Bonferroni posttest was used to assess statistical differences between groups tested. $P<.05$ was considered to represent a significant difference in both analyses used. For *in vitro* results, one-way ANOVA was used followed by Bonferroni posttest. $P<.05$ was considered to represent a significant difference in both analyses used. All data were expressed as mean ± S.E.M.

3. Results

Firstly, we determined the glucose blood levels 7 days after STZ administration. The rats with fasting glucose ≥250 mg/dl were separated to start the treatment (data not shown). After 30 days of treatment, the blood was collected, the serum was separated, and the plasmatic glucose was determined. In Table 2, the glucose blood levels after 30 days of study are shown. The glucose levels in the diabetic group were significantly increased when compared to the control group. The treatment with chlorogenic acid (5 mg/kg), caffeine (15 mg/kg) and coffee (0.5 g/kg) showed no effect on decreasing the increased glucose levels.

We analyzed the fructosamine levels since it is used for estimating the degree of protein glycation in diabetes. Results of the fructosamine levels are presented in Table 2. There was an increase of 68% in

Table 2
Glucose levels of control and STZ-induced diabetic rats and treated with CGA, CA and CF at the end of treatment (n=10)

Groups	Glucose(mg/dl)	Platelets(n*10^6/mm^3)	Fructosamine (mmol/L)
Control (WT)	90.53±2.7	0.72±0.12	183±13.9
Control (CGA)	85.3±8.84	1.07±0.13	200±9.57
Control (CA)	79.1±7.3	0.74±0.2	190±9.57
Control (CF)	89.46±8.31	0.62±0.17	205±5.77
Diabetic (WT)	258.5±15.75 *	0.94±0.16	309.4±23.47 *
Diabetic (CGA)	251.3±13.65 *	0.92±0.11	292.5±43.04 *
Diabetic (CA)	235.2±16.4 *	0.60±0.1	275.0±30
Diabetic (CF)	258.3±19.3 *	0.65±0.14	243.8±18.19

Platelet number in plasma-rich platelets of STZ-induced diabetic rats and treated with CGA, CA and CF (n=5). Serum fructosamine levels (μmol/L) of STZ-induced diabetic rats and treated with CGA, CA and CF. Bars represent means±S.E.M. One-way ANOVA was followed by Bonferroni posttest.
* Significant difference in relation to the control (WT) group, at P<.05, n=10.

fructosamine levels in the diabetic (WT) group when compared to the control group. Treatments were not able to reduce such increase.

Then, we chose to study the effects of these compounds on certain parameters of the purinergic system in the CNS. We evaluated the total nucleotides concentration in the cerebral cortex by HPLC. ATP (A), ADP (B), AMP (C) and the nucleoside adenosine (D) concentrations are shown in Fig. 1. Statistical analysis showed a significant increase in ATP (64.5%) and ADP (42%) concentrations in the diabetic (WT) group when compared to the control (WT) group. No alterations of AMP or adenosine concentrations were observed when compared to the control (WT) group. Interestingly, in the diabetic (CF) group, a significant increase in ATP (54%) and ADP (58%) concentrations was observed when compared to the control (WT) group.

The levels of these molecules are controlled through a family of enzymes, denominated ectonucleotidases. Then, we determined the NTPDase and 5′-nucleotidase activity in the synaptosomes from cerebral cortex. Those results are shown in Fig. 2. Statistical analysis demonstrated that NTPDase activity when ATP (Fig. 2A) and ADP (Fig. 2B) were used as substrate did not show any differences between tested groups. Only coffee treatment showed an increase (51%) in ADP hydrolysis in the diabetic (CF) group when compared to the control (WT) group. In addition, two-way ANOVA statistical analysis showed a significant control or diabetic×water or treatment interaction for ADP hydrolysis [F(3,66) = 2.88; P=.0421]. In relation to 5′-nucleotidase, two-way statistical analysis showed a significant control or diabetic×water or treatment interaction for AMP hydrolysis [F(3,61) = 7,46; P=.04] (Fig. 2C). *Post hoc* analysis revealed that 5′-nucleotidase activity was significantly increased in the diabetic (WT) groups (173%) and diabetic (CF) group (383%) when compared to the control (WT) group.

In addition, we analyzed the expression of the ectonucleotidases to determine if hyperglycemia could change its expression before changing the activity in the cerebral cortex of diabetic rats. Fig. 3 shows that there were no alterations of NTPDase1 (A), NTPDase2 (B), NTPDase3 (C) or 5′-nucleotidase (D) expression in cerebral cortex of STZ-induced diabetic rats.

Also, as we finalize the CNS analysis, we performed an *in vitro* assay to show the capacity of chlorogenic acid, caffeine and both together (simulating a coffee effect) to affect the NTPDase and 5′-nucleotidase activity in synaptosomes from cerebral cortex. These results are presented in Fig. 4. As it can be observed, there was a decrease in NTPDase activity when ADP was used as substrate at 100–200 μM of chlorogenic acid. Similarly, when AMP was used as substrate, an inhibition of 5′-nucleotidase activity was found at 50–200 μM of chlorogenic acid. The addition of caffeine to the incubation medium did not alter NTPDase and 5′-nucleotidase activity. The combination of chlorogenic acid and caffeine produced a significant activation in AMP hydrolysis at 150- and 200-μM concentrations.

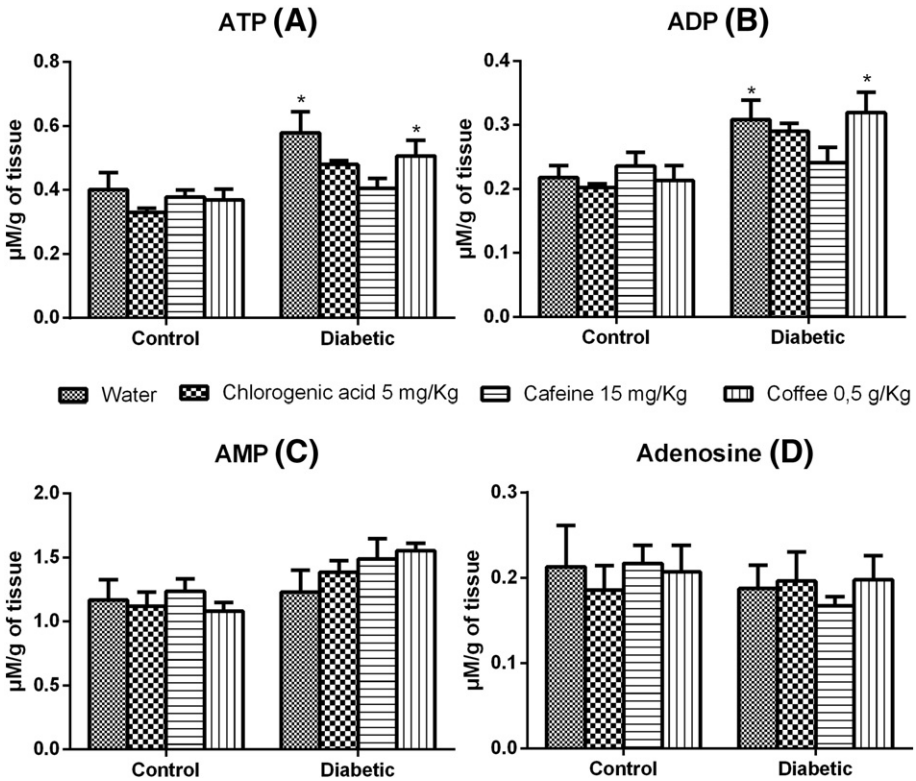

Fig. 1. Quantification of nucleotides ATP (A), ADP (B), AMP (C) and adenosine (D) in the cerebral cortex of control and STZ-induced diabetic rats and those treated with WT, CGA, CA and CF. Bars represent means±S.E.M. One-way ANOVA was followed by Bonferroni posttest. *Significant difference in relation to the control group, at P<.05, n=4.

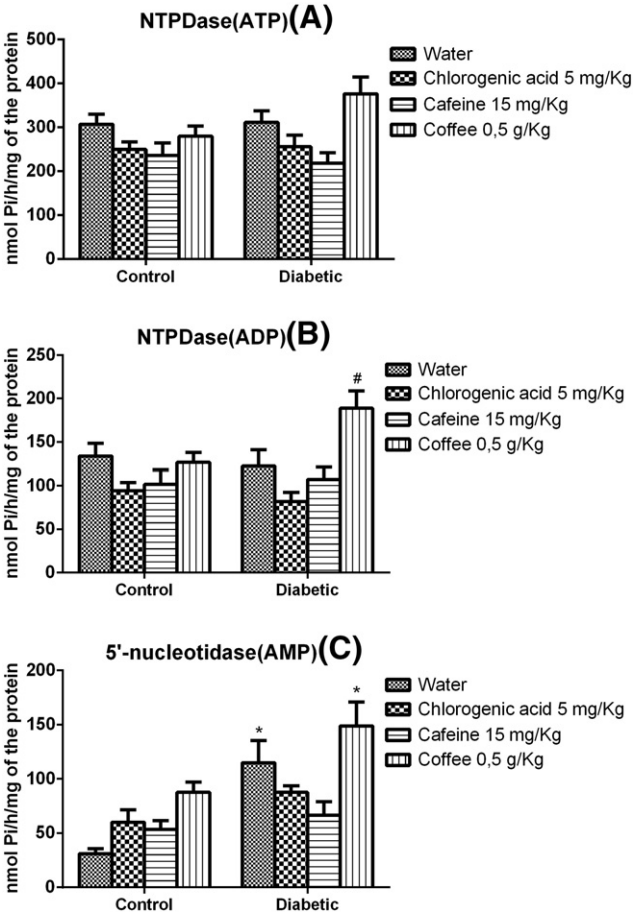

Fig. 2. NTPDase and 5′-nucleotidase activity in synaptosomes of the cerebral cortex of control and STZ-induced diabetic rats and those treated with WT, CGA, CA and CF using ATP (A), ADP (B) and AMP (C) as substrate (nmol Pi/min/mg of protein). Bars represent means±S.E.M. One-way ANOVA was followed by Bonferroni posttest. *Significant difference in relation to the control group, at $P<.05$, $n=6–8$.

Furthermore, we decided to investigate the effects of chlorogenic acid in the platelets. The platelets were selected only because hyperglycemia alters their functions and promotes the thrombus formation, which contributes to macrovascular complications found in the diabetes. Table 2 presents the results obtained for the plasma platelet numbers. All groups tested demonstrated no alterations in such parameter.

Fig. 5 shows the results obtained for platelet aggregation using ADP as agonist at 5-μmol (A) and 10-μmol (B) concentrations. Two-way ANOVA statistical analysis showed control or diabetic×water or treatment interaction for 10 μmol ADP [$F(3,22) = 11.3$; $P=.0001$]. Furthermore, an increase in platelet aggregation of 72% at 5-μmol ADP and 88% at 10-μmol ADP concentration was observed in the diabetic (WT) group when compared to the control (WT) group. Treatment with chlorogenic acid significantly decreased platelet aggregation in 58% and 66.0% in the diabetic (CGA) group when compared to the diabetic (WT) group at both 5-μmol and 10-μmol agonist concentrations, respectively. Treatment with coffee significantly decreased platelet aggregation in the diabetic (CF) group in 43% and 66% when compared to the diabetic (WT) group at both 5-μmol and 10-μmol agonist concentrations, respectively.

Then, we pondered if these changes could be due to alterations in NTPDase and 5′-nucleotidase activity since these enzymes regulate ATP, ADP and adenosine levels, which are key molecules in the control of platelet aggregation. Two-way ANOVA statistical analysis showed a significant control or diabetic×water or treatment interaction for ATP hydrolysis [$F(3,58) = 3.0$; $P=.04$] (Fig. 6A) and for ADP hydrolysis [$F(3,51) = 8.3$; $P=.0001$] (Fig. 6B). Furthermore, NTPDase activity was increased in ATP (69%) and ADP (136%) hydrolysis in the diabetic (WT) group when compared to the control (WT) group. *Post hoc* analysis revealed that the treatment with chlorogenic acid decreased ATP and ADP hydrolysis (49% for ATP and 65% for ADP), as did caffeine (56% for ATP and 62% for ADP), and coffee decreased ATP and ADP hydrolysis (52% for ATP and 49% for ADP) in the diabetic (CGA), (CA) and (CF) groups, respectively, when compared to the diabetic (WT) group ($P<.001$). The results obtained for 5′-nucleotidase (Fig. 6C) activity were different from those found for NTPDase activity. An increase in AMP hydrolysis was observed in the diabetic (WT) (191%) and diabetic (CGA) (212%) groups when compared to the control (WT) group.

Posteriorly, we analyzed if those compounds *in vitro* could alter the NTPDase and 5′-nucleotidase activity in platelets. The same experimental design was used for the platelets and cerebral cortex. Chlorogenic acid inhibited the hydrolysis of ATP, but not of ADP or AMP, when PRP was incubated at 50–200-μM concentration (data not shown). In addition, no significant alterations in nucleotide hydrolysis were observed when caffeine and chlorogenic acid plus caffeine were incubated with PRP.

4. Discussion

Studies of our research group have reported that the purinergic system can be altered in different pathologies such as lung cancer [36], acute myocardial infarction [37], hypertension [38], hypoxia–ischemia [39], impairment of memory [8] and DM [14]. In this study, we found an increase in 5′-nucleotidase activity in cerebral cortex synaptosomes. An increase in the ATP and ADP levels in the cerebral cortex of the diabetic rats was found when compared with control rats. In addition, no changes were observed in NTPDase and 5′-nucleotidases expression in the cerebral cortex.

The cerebral cortex is highly sensitive to glucose fluctuations, and perhaps, it may be more affected by alterations promoted by hyperglycemia [40]. Moderate cognitive impairment is reported and cortical atrophy is found in these patients. Furthermore, high glucose levels lead to an increased level of glucose in the brain, increasing sorbitol levels and affecting protein kinases activity, oxidative stress and formation of AGEs contributing with neuron damage [41]. In addition, the lack of insulin or problems in signaling the insulin receptor in the CNS are important because insulin is a modulator involved in several signaling in the brain, including glucose homeostasis and cognition [42]. Glucose metabolism in the brain remains the primary means to generate the large amount of energy required to fuel synaptic action and ionic homeostasis [43]. It is possible to suggest that ATP accumulation could occur due to excitotoxicity of the neuron [44] or due to decrease detected in Na^+, K^+-ATPase activity, which it is found to be inhibited in DM [23]. All these results might signify damage in the CNS, which corroborate with existing data obtained in STZ-induced diabetic in rats [23,44].

The alterations in memory, learning and cognition in diabetes can be modest, but occasionally, they can be severe [41]. The difference between damage levels is correlated with time of diabetes progression since milder changes are observed at 20 days after diabetes induction [45] and, at 10 weeks, more intense cognitive alterations are observed [41,42]. It is possible to explain it because the NTPDase and 5′-nucleotidase did not change their cDNA expression. Cardoso et al. (2010) demonstrated that some parameters, including antioxidant parameters, are altered in prediabetic Wistar rats (glucose level was 6.6 mmol/L), while others remain unchanged [42]. Lunkes et al. (2008) showed that NTPDase and 5′-nucleotidase activities increase with an increase in glucose and fructose concentration [13]. In addition, the glucose blood levels of diabetic rats shown in our results were

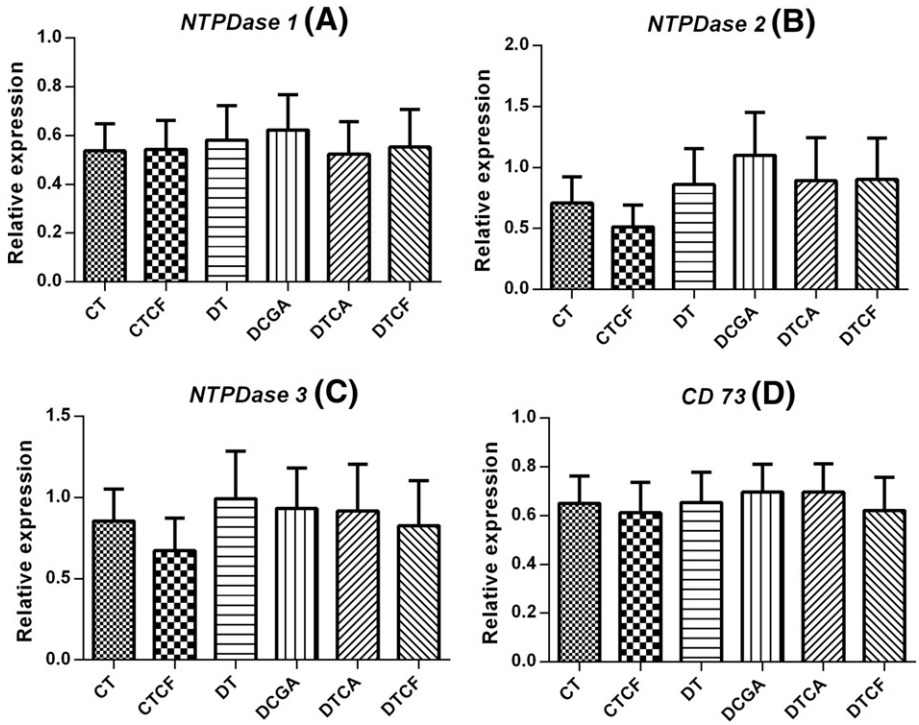

Fig. 3. NTPDase1 (A), NTPDase2 (B), NTPDase3 (C) and 5′-nucleotidase expression in the cerebral cortex of control and STZ-induced diabetic rats and those treated with WT, CGA, CA and CF. Bars represent means±S.E.M. One-way ANOVA at $P<.05$, $n=4$.

increased, but it is possible that they were not increased enough to make changes in the expression of these enzymes since NTPDase activity also remained unchanged.

Although NTPDase activity did not change, an increase in 5′-nucleotidase (5′-NT) activity was observed. This could have occurred because enzymes other than those measured here, such as pyrophosphatase/phosphodiesterases and alkaline phosphatases [11], can hydrolyze ATP directly to AMP. An increase in 5′-NT would produce high adenosine levels, but it has been demonstrated that there is an increase in the adenosine deaminase (ADA) activity in DM [9,46]. In these conditions, the adenosine is rapidly removed by ADA in the extracellular environment. Corroborating these findings, in our experiment, adenosine levels in the brain were not found altered in diabetic rats, which could indicate that adenosine could not fulfill its protective effect against excitotoxicity [44].

The life span of neurons is quite different from platelets. Normally, platelet life span is just 5 to 9 days. It is possible to suggest that platelets are more sensitive than neurons when harmful compounds are circulating in the body. Differently from the synaptosomes from the cortex cerebral, we observed that the diabetic rats showed an increase in platelet aggregation and, consequently, an increase NTPDase and 5′-NT activity. Furthermore, high glucose levels could promote NTPDase glycosylation, leading to its increased activity [47]. Glycosylation of proteins can cause structural changes altering their function and, consequently, can induce injury in the blood vessels. Markers used for estimating the degree of protein glycation in diabetes include fructosamine and glycated hemoglobin levels [48]. Our study demonstrated that fructosamine levels were increased in the STZ-induced diabetic rats and could indicate an increase in protein glycosylation.

In addition, high concentrations of glucose similar to those found in hyperglycemia status increase *per se* NTPDase and 5′-NT activity *in vitro* in platelets [13]. The increase observed in ATP and ADP hydrolysis by NTPDase could be one mechanism of decreasing the concentration of these nucleotides since ADP and ATP can increase platelet aggregation [49]. Lohman et al. reported that it has been suggested that chronic increases in ATP in the vasculature could potentiate pathological conditions such as hypertension and atherosclerosis [50]. It is possible that increases in ATP occur in the hyperglycemic state and contribute to an increase in NTPDase activity and platelet aggregation. Also, high AMP levels can increase the activity of 5′-NT, leading to an increase in levels of adenosine, which could act as a protective molecule by decreasing platelet activation as a compensatory mechanism [51]. However, adenosine levels could be reduced once ADA activity is found increased in serum [9], erythrocytes [52] and platelets [15]. In these terms, adenosine in not enough to protect the vascular system from damage promoted by hyperglycemia.

Our results demonstrated that coffee treatment increased ADP and AMP hydrolysis in synaptosomes. Similar results were also observed when diabetic rats were treated with resveratrol [51]. The increase in enzymatic activity would occur due to the increase in nucleotide concentration observed in diabetic rats treated with coffee. Furthermore, when we tested the *in vitro* effects of the combination of chlorogenic acid and caffeine on NTPDase and 5′-NT activity in synaptosomes, we observed a different effect when compared to chlorogenic acid and caffeine separately. Farah and Danangelo mentioned that chlorogenic acid could be associated with the caffeine in the coffee, justifying a different effect obtained *in vivo* with coffee and caffeine in combination with chlorogenic acid [53]. Therefore, the increase in ADP hydrolysis in synaptosomes could be associated with numerous compounds contained in this beverage since the combination of chlorogenic acid plus caffeine did not increase ADP hydrolysis in *in vitro* tests. It is possible that these compounds can cross the blood–brain barrier and act on an NTPDase isoform. Thus, it is difficult to determine the compounds or the synergy among them responsible for these effects.

Unlike in synaptosomes, a decrease in NTPDase and 5′-NT activity in platelets was observed in diabetic rats treated with coffee when

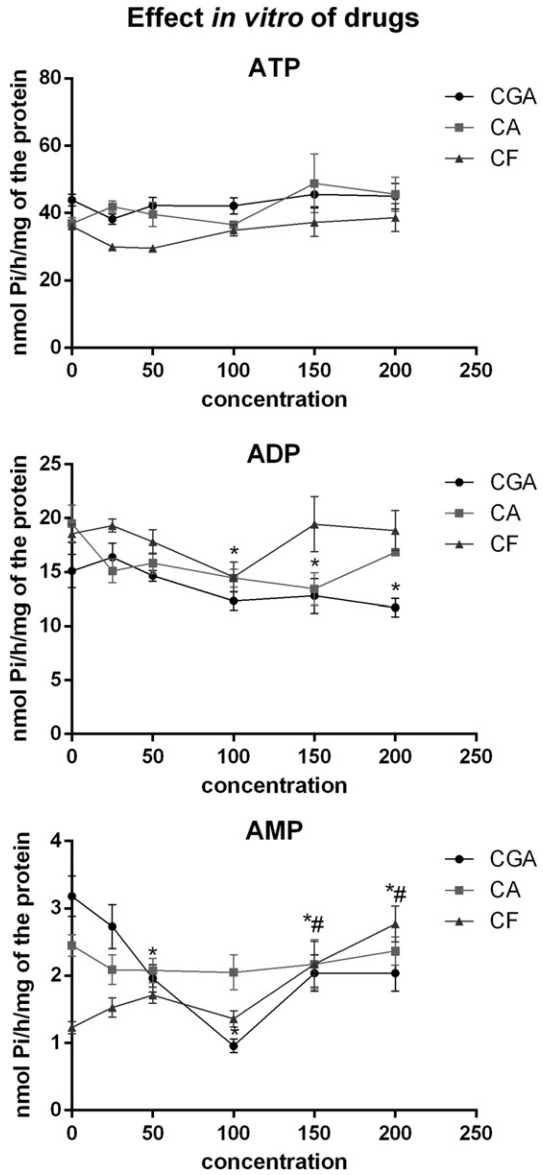

Fig. 4. *In vitro* effects of CGA, CA and CGA+CA on NTPDase and 5′-nucleotidase activity in synaptosomes from the cerebral cortex of control rats. Results are expressed in means± S.E.M. Groups with * had significantly decreased activity and those with # had significantly increased activity when compared to the control (0 concentration) ($P<.05$, $n=5$; one-way ANOVA–Bonferroni test).

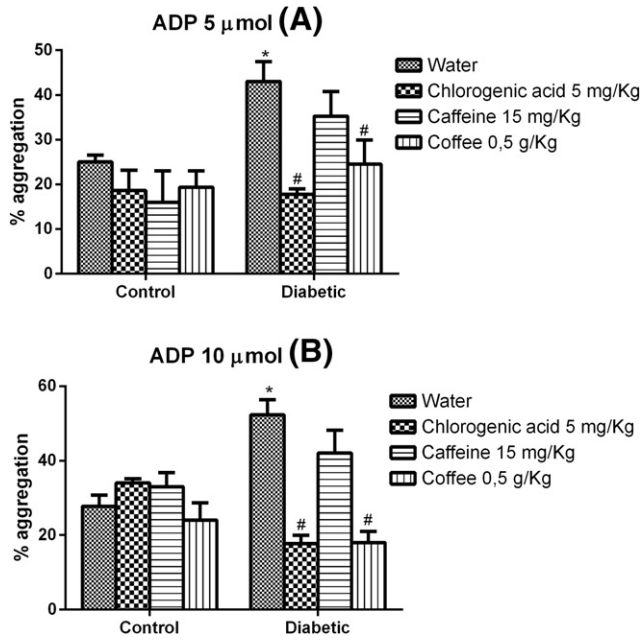

Fig. 5. Percentage platelet aggregation using agonist ADP at 5 μmol (A) and 10 μmol (B) in plasma-rich platelets from control and STZ-induced diabetic rats and those treated with WT, CGA, CA and CF. Bars represent means±S.E.M. One-way ANOVA was followed by Bonferroni posttest. *Significant difference in relation to the control group; #significant difference in relation to the diabetic control group, at $P<.05$.

compared to diabetic (WT) rats. It is important to note that the coffee treatment was able to reduce platelet aggregation in our study. Natella et al. showed that coffee consumption reduces platelet aggregation in healthy volunteers when they used collagen and arachidonic acid as aggregate molecules after 30 and 60 min of coffee consumption, respectively [54]. Together with our results obtained about the effect on aggregation using ADP as an agonist, we suggest that coffee can act as a platelet antiaggregation agent. Coffee is rich in phenolic compounds and other substances, and these compounds have effects on scavenging ROS as well as on decreasing AGE formation [55,56]. Moreover, Natella and collaborators showed that a mix of phenolic acids from coffee was able to significantly inhibit platelet aggregation and collagen-induced thromboxane B2 formation *ex vivo* in healthy subjects. It is important to note that diabetic rats treated with coffee showed a 21% decrease in fructosamine levels. Verselloni at al. demonstrated that coffee fractions, mainly the ones that have a high concentration of chlorogenic acid, showed an antiglycant effect and may decrease alterations caused by high glucose concentration [56–58]. In this context, coffee may have a protector effect on the cardiovascular system in the diabetic state, attributed to chlorogenic acid, due to its reduction of platelet aggregation that is a factor in cardiovascular disease promotion.

Caffeine is a methylxanthine and the major component present in coffee. Our results showed that caffeine could decrease NTPDase activity in platelets in the diabetic group treated with caffeine. However, 5′-NT activity in platelets and platelet aggregation did not present changes during treatment with caffeine. A classical effect of caffeine is like adenosine receptor antagonists, mainly A_1 and A_2 receptors, which may affect the neuromodulation carried out by adenosine [59,60] and platelet aggregation [16]. On the other hand, xanthine compounds may also inhibit platelet aggregation by inhibiting platelet-activating factor, which can decrease the release of ATP [61], but could not act when ADP is the agonist, as demonstrated by our results. When there is an increase in the release of ATP and ADP in the extracellular medium, NTPDase activity can increase as compensation in order to reduce aggregation molecules.

It is well known that natural compounds such as chlorogenic acid can act in preventing the progression of diabetic complications by decreasing hyperglycemia [62]. Chlorogenic acid may protect neurons from oxidative effects; this compound protects the cerebral cortex from the reduction of δ-ALA-D and Na^+, K^+-ATPase activity, memory loss and increase in AChE activity in diabetic rats [23]. However, the concentration that reaches the brain could not be enough to protect alterations in the purinergic system arising from diabetes. In addition, Farah et al. confirmed that chlorogenic acid in coffee is absorbed and metabolized in the whole gastrointestinal tract and can be found in high levels in the plasma of subjects that ingested chlorogenic acid from green coffee [63], but whether chlorogenic acid could be found in the brain in high concentrations is still unknown.

Our results demonstrated a decrease in platelet aggregation as well as a decrease in NTPDase activity (when ATP and ADP were used as substrate) in platelets in the diabetic group treated with 5 mg/kg chlorogenic acid when compared to the diabetic (WT) group.

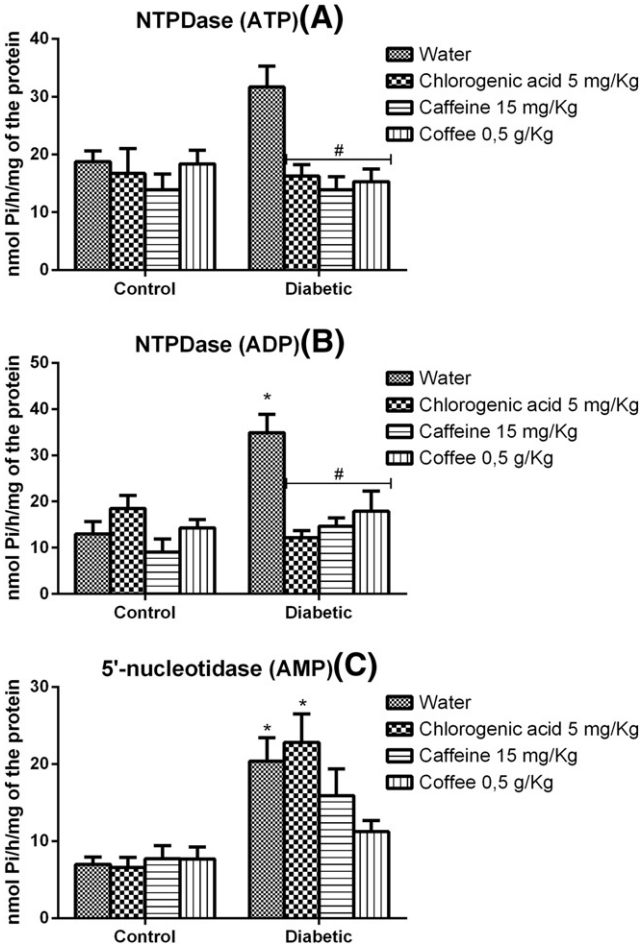

Fig. 6. NTPDase and 5′-nucleotidase activity in platelets from control and STZ-induced diabetic rats and those treated with WT, CGA, CA and CF using ATP (A), ADP (B) and AMP (C) as substrate (nmol Pi/min/mg of protein). Bars represent means±S.E.M. One-way ANOVA was followed by Bonferroni posttest. *Significant difference in relation to the control group; #significant difference in relation to the diabetic control group, at $P<.05$.

Furthermore, *in vitro* results showed that ATP hydrolysis in platelets could be inhibited by chlorogenic acid. Moreover, an increase in the hydrolysis of AMP by 5′-NT could be observed in the diabetic (CGA) group; that result could indicate a beneficial effect on the organism due to the increased production of adenosine, which in turn may exert its antiplatelet activity and control the levels of ATP and ADP. In addition, Fuentes et al. (2014) showed that CGA increases the cyclic AMP level, which is responsible for inhibiting the platelet aggregation. Such effect may be compatible with activation of the A_{2A} receptor [64], and probably, the augmented adenosine level seems to be responsible for the reduced platelet aggregation found in response to chlorogenic acid treatment.

In this context, chlorogenic acid seems to act on the purinergic system. However, more investigation is required to promote a better understanding. Our research group has been working with this compound and testing its beneficial effects against diabetes in different tissues [23].

In conclusion, the results of the present study demonstrated that hyperglycemia promoted alterations at molecular levels more prominent in the blood than the CNS in 30 days of STZ-induced diabetes. In addition, treatment with chlorogenic acid, caffeine and coffee can modulate the hydrolysis of adenine nucleotides and nucleosides and could contribute to protect mainly platelets against the dangerous effects of DM. These results emphasized the influence of this polyphenol and other antioxidants in the diet for human health, possibly preventing disorders associated with hyperglycemia. Future studies will analyze the effect of chlorogenic acid in relation to diabetic damage and try to discover the mechanisms by which chlorogenic acid might act to protect cells against hyperglycemia damage.

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