Original Paper

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2010;26:729-738

Accepted: September 14, 2010

Syzygium cumini Extract Decrease Adenosine Deaminase, 5'Nucleotidase Activities and Oxidative Damage in Platelets of Diabetic Patients

Karine S. De Bona[1], Luziane P. Bellé[2], Marcel H. Sari[1], Gustavo Thomé[3], Maria R.C. Schetinger[3], Vera M. Morsch[3], Aline Boligon[1], Margareth L. Athayde[1], Aline S. Pigatto[4] and Maria B. Moretto[1]

[1]Postgraduate Program in Pharmaceutical Sciences, Health Science Centre, Federal University of Santa Maria (UFSM), [2] Postgraduate Program in Pharmacology- UFSM -Santa Maria, [3]Department of Chemistry - UFSM -Santa Maria, [4]Franciscan University Center–UNIFRA-Santa Maria

Key Words

Adenosine deaminase • Diabetes mellitus • 5'Nucleotidase • Oxidative stress • Platelets • *Syzygium cumini* leaves extract

Abstract

Diabetes mellitus, a chronic metabolic disorder, has assumed epidemic proportions and its long-term complications can have devastating consequences. The oxidative stress in diabetes was greatly increased due to prolonged exposure to hyperglycemia and impairment of oxidant/antioxidant equilibrium. *Syzygium cumini* is being widely used to treat diabetes by the traditional practitioners over many centuries. Adenosine deaminase (ADA) and 5'-Nucleotidase (5'NT) are enzymes of purine nucleoside metabolism that play an important role in the regulation of adenosine (Ado) levels. In this study, we investigated the effect of *Syzygium cumini* aqueous leaves extract (ASc) on ADA and 5'NT activities and on parameters of oxidative stress under *in vitro* conditions, using platelets of patients with Type 2 diabetes mellitus. Platelet-Rich Plasma (PRP) was assayed by ADA, 5'NT, Catalase (CAT), Superoxide Dismutase (SOD) activities and Thiobarbituric acid reactive substances (TBARS) levels. We observed that ADA, 5'NT activities and TBARS levels were significantly higher when compared to the control group, and ASc (100 and 200 µg/mL) prevented these effects. Our study demonstrates that ASc was able to remove oxidant species generated in diabetic conditions and modulates in the Ado levels. Then, ASc may promote a compensatory response in platelet function, improving the susceptibility-induced by the diabetes mellitus.

Copyright © 2010 S. Karger AG, Basel

Introduction

Diabetes mellitus (DM) is a worldwide health problem predisposing to markedly increased cardiovascular mortality and morbidity [1, 2].The prevalence of type 2 DM continues to increase globally and brings a parallel increase in complications associated with cardiovascular disease [3-5]. Indeed, individuals with type 2 DM have a 2- to 4-fold higher risk of developing atherosclerotic cardiovascular disease [6]. Given the myriad perturbations of endothelial dysfunction,

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2010 S. Karger AG, Basel
1015-8987/10/0265-0729$26.00/0

Accessible online at:
www.karger.com/cpb

Maria Beatriz Moretto
Postgraduate Program in Pharmaceutical Sciences
Health Science Centre, Federal University of Santa Maria (UFSM)
97105-900 - Santa Maria, RS (Brazil)
Fax +55-3220 8018, E-Mail Beatriz@smail.ufsm.br

inflammation, thrombosis, oxidative stress, dyslipidaemia associated with DM, platelet function plays an important role in the long-term development of vascular disease in these patients.

Platelets are responsible for maintaining vascular integrity [7]. Vascular disease associated with altered vascular reactivity is a major complication of DM [8]. Platelets from diabetic patients show greater adhesion and aggregation [9]. Also, blood platelets generate reactive oxygen/nitrogen species (ROS/RNS) that can be involved in regulation and modulation of their function [10-12]. Moreover, increased free radical activity is suggested to play an important role in lipid peroxidation and protein oxidation of cellular structures causing cell injury and is implicated in the pathogenesis of vascular disease in type 2 DM [13-15]. Platelets are also a very good model for the study of oxidative stress. Oxidative stress reflects an unfavorable imbalance between potentially harmful oxidants and protective antioxidants leading to altered cellular redox state [16]. ROS/RNS have been reported to modulate and, as second messengers, regulate the physiological response of blood platelets (which are involved in hemostasis) to different stimuli [17, 12]. Intracellular sources of ROS in activated platelets are the arachidonic acid pathway (via cyclooxygenase or 12-lipoxygenase) stimulated by different agonists, the GSH cycle [18, 11], and metabolism of phosphoinositides [12].

Platelets express several enzymes on their surface, which constitute a highly organized enzymatic cascade. These enzymes are able to regulate the extracellular concentrations of adenine nucleotides and nucleosides and play an important role in the maintenance of normal hemostasis and thrombogenesis, mainly by regulating the platelet aggregation status [19-21]. One of these enzymes is Adenosine deaminase (Adenosine aminohydrolase, EC 3.5.4.4, ADA) that is one of the key enzymes of purine nucleoside metabolism, participating in the conversion of adenosine (Ado) to inosine and 2'-deoxyadenosine (dAdo) [22]. Two different isoenzymes of ADA designated as ADA1 and ADA2 have been found in mammals and in lower vertebrates. The ADA1 isoenzyme is found in all cells, with the highest activity in lymphocytes and monocytes, whereas ADA2 is the predominant isoenzyme in the serum of normal subjects [23]. ADA is suggested to be an important enzyme for modulating the bioactivity of insulin [24] and in the acute and protracted inflammatory responses [25, 26].

Recent studies have shown that ADA activity was higher in the brains of young rats, when compared to the 60-day-old adult rats [27]. Furthermore, observed an increase in the activity of ADA in the cerebral cortex of rats eight days after neonatal Hypoxia Ischemia [28] and the administration of Metothrexate (MTX) induced a significant decreased in the ADA activity in the cerebral cortex, kidney and liver tissues of young rats [29]. Besides, ADA activity is decreased in plasma of rats exposed to aged and diluted sidestream smoke [30], and in lymphocytes and platelets of patients with multiple sclerosis [31, 32].

The enzyme ecto-5'-Nucleotidase (E.C. 3.1.3.5, CD73, 5'NT) is located in the platelet membrane and promotes the hydrolysis of AMP to its nucleoside, adenosine, in the extracellular medium [33, 34]. Recent studies report that 5'NT is altered in type 2 DM and hypertensive patients probably related with the thromboregulation process [35, 36]. The product of 5'NT, adenosine, is an important modulator of vascular tone, and it is a well-known inhibitor of platelet aggregation [37, 38].

Plant infusions and decoctions have been used as popular medicines in several underdeveloped and developing countries as an alternative treatment for various conditions, including DM [39]. Among these medicinal plants, *Syzygium cumini* (L.) Skeels (sin.: *Eugenia jambolana* Lam., *Syzygium jambolanum* DC) of family Myrtaceae has been intensively studied as an antidiabetic agent and is often recommended as an adjuvant for treatment of type 2 DM. Most of the people prepared an infusion or decoction of dry leaves in an average dilution of 2.5 g/L (0.2–6.9) for *Syzygium cumini* [40]. In other studies, each patient received 2.0 g/day of dry leaf to prepare 1 L of tea [41]. It has been demonstrated that the bark, fruits, seeds or leaves of this plant collected from various regions of the world and administered in different pharmaceutical preparations (e.g. tinctures and aqueous extracts) decrease blood glucose levels in diabetic animals [42] and that polyphenolic compounds, including flavonoids and phenolic acids have various physiological functions, such as antioxidant, antihyperglycemic and antihypertensive properties [43].

Previous findings have reported alterations in 5'NT and ADA activity in platelets from Streptozotocin-induced diabetic rats [21]. Recently, we demonstrated that ADA activities were higher in serum and erythrocytes of hyperglycemic subjects than in control subjects [44]. Both enzymes modulate the concentration of adenosine that can affect the platelets function and contribute for the pathogenesis of the vascular complications. Also, we observed that *Syzygium cumini* leaves extract (ASc)

inhibited ADA activity and reduced glucose levels in hyperglycemic patients [44]. In this regard, due to the significant properties in the hyperglycemic conditions and the meaning of enzymes that hydrolyzes adenine nucleotides in the mechanism of thromboregulation, the present study was undertaken to evaluate the effect of ASc on ADA and 5'NT activities and oxidative stress parameters in platelets from type 2 diabetics under *in vitro* conditions. Finally, we to aim to identify the isoforms of ADA present in these platelets, using the Erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), the well-establishment inhibitor of ADA 1.

Materials and Methods

Chemicals

Adenosine was obtained from Merck (Darmstadt, Germany). The substrate AMP was obtained from Sigma Chemical Co (St. Louis, MO, USA) and bovine serum albumin from Reagen (Paraná, Brazil). All other chemicals were of analytical grade and were obtained from standard commercial suppliers.

Plant material and aqueous leaves extract of Sygyzium cumini (L.) Skeels (ASc) preparation

Leaves of *Syzygium cumini* were freshly locally collected, cleaned, dried, and powdered. They were identified by the Laboratory of Botanic and Pharmacognosy of the Franciscan University Center – UNIFRA - Santa Maria, RS, Brazil. The leaves were dried in a greenhouse / (renewal of air) at 40°C for approximately 48 h. Then, they were smashed in the knife mill. The products were submitted to extraction with ethanol 80% in a Soxhlet apparatus until exaustion. After extraction, the solvent was evaporated by a rotavapor, supplying the crude extract. The mother solution 1% was made from the crude extract dissolving 1g of this extract in 100ml of 0.9% NaCl solution [45].

High-performance liquid chromatography (HPLC) characterization of the extract

Chromatographic analyses were carried out in isocratic conditions using RP-C_{18} column (4.6 mm x 250 mm) packed with 5 µm diameter particles. The mobile phase was methanol-acetonitrile-water (40:15:45, v/v/v) containing 1.0% acetic acid. The flow rate was 0.8 mL/min, injection volume 20 µl and the wavelength 257 nm. The mobile phase was filtered through a membrane filter 0.45 µm and then degassed by an ultrasonic sound before use. The solutions of standards (rutin, kaempferol, chlorogenic acid and caffeic acid) were prepared in the same mobile phase of HPLC to the standard curve in the concentration range of 0.0125 to 0.200 mg/ml. The chromatographic peaks were confirmed by comparing its retention time with those of reference standards and quantification was performed by peak integration using the external standard method. The calibration curve for chlorogenic acid was: Y = 30153x – 214576 (r = 0.9998), the curve of gallic acid was: Y = 16324x – 661582 (r = 0.9967), and the curve of rutin was: Y= 19217x – 16949 (r = 1). All chromatographic operations were performed at room temperature and in triplicate [46].

Sample collection and preparation

The study was in accordance with the guidelines of the Ethics Committee of the Federal University of Santa Maria which approved the experimental protocol (23081.004068-76). Blood samples were taken from 30 patients (19 men and 11 women) with type 2 DM who have done routine tests at the Center of Diagnosis and Secondary Support (Centro de Diagnóstico e Apoio Secundário - CEDAS – Santa Maria – RS/ Brazil). The mean (± SE) age of the patients was 56.83 ± 2.13 years old and fasting glucose levels were 169.9 (±10.26) mg/dL. Data such as age, medication use, presence of malignancy or other status were obtained through the requirements brought by patients at the time of the blood collection. These patients did not use Non steroidal anti inflammatory drug (NSAID) and antiplatelet drugs. Alcoholic subjects, pregnant women, and people with cancer were excluded from the study. The control group consisted of 17 healthy subjects (11 men and 6 women).

Platelet-Rich Plasma (PRP) was prepared by the method of Pilla [31] with the following minor modifications. Total blood was collected with 0.120 M sodium citrate as anticoagulant. The total blood–citrate system was centrifuged at 160 xg during 15 min. Next, the PRP was centrifuged at 1400 xg for 30 min and washed twice with 3.5 mM HEPES buffer, pH 7.0, containing 142 mM NaCl, 2.5 mM KCl and 5.5 mM glucose. Platelet pellets were resuspended in HEPES buffer and used to determine enzymatic activities. Biochemical and hematological parameters were determined using routine clinical chemical assays.

5'-NT from platelet assay

The ASc was added to the incubation medium at the following concentrations 100 and 200 µg/mL. Enzymatic assay was carried out in a reaction medium containing 10 mM $MgCl_2$, 100 mM NaCl, 5 mM KCl, 6 mM glucose and 50 mM Tris–HCl buffer, pH 7.4, at a final volume of 200 µl as described by Lunkes [35]. Twenty microliters of the enzyme preparation (8–10 µg of protein) was added to the reaction mixture and the pre-incubation proceeded for 10 min at 37°C. The reaction was initiated by the addition of AMP at a final concentration of 2.0 mM, and the time of incubation was 60 min. The reaction was stopped by the addition of 200 µl of 10% trichloroacetic acid (TCA) to provide a final concentration of 5%. After this, the inorganic phosphate released by AMP hydrolysis was determined in triplicate by the method of Chan [47] using KH_2PO_4 as standard. The same process was carried out on the control tubes to exclude non enzymatic hydrolysis, by adding twenty microliters of protein to the reaction medium after TCA. The results were expressed as nmol inorganic phosphate released/ minute/ milligram of protein (nmol Pi released/ min/ mg protein).

ADA activity

ADA activities were measured spectrophotometrically in platelets using the method of Giusti and Gakis [48]. ASc was

	Control	type 2 DM
Age (years)	53.94 ± 3.2	56.83 ± 2.13
♂/♀	11 / 6	19 / 11
HbA1c (g%)	5.98 ± 0.21	**8.06 ± 0.43
Glucose (mg/dL)	91.84 ± 1.81	***169.9 ± 10.26
Urea (mg/dL)	38.2 ± 2.41	36.5 ± 2.5
Creatinine(mg/dL)	0.95 ± 0.06	0,9 ± 0.03
Triglycerides (mg/dL)	128.1 ± 12.15	**252.6 ± 32.7
Total Cholesterol(mg/dL)	204.6 ± 9.4	209.9 ± 10.34
HDL-C (Mg/dL)	47.16 ± 4.6	46.47 ± 1.6
Hemoglobin (g/dL)	14.30 ± 0.5	14.0 ± 0.3
Hematocrit (%)	43.28 ± 1.32	42.05± 0.72
Platelets (n/mm3)	223.200 ± 12.31	251.300± 11.86

Table 1. Biochemical and hematological parameters of the experimental groups: Data are presented as mean ± SEM. HbA1c= glycosylated hemoglobin; HDL-C=high density lipoprotein cholesterol. Statistically significant differences from controls, as determined by Student's t-test (**p < 0.01; ***p < 0.001).

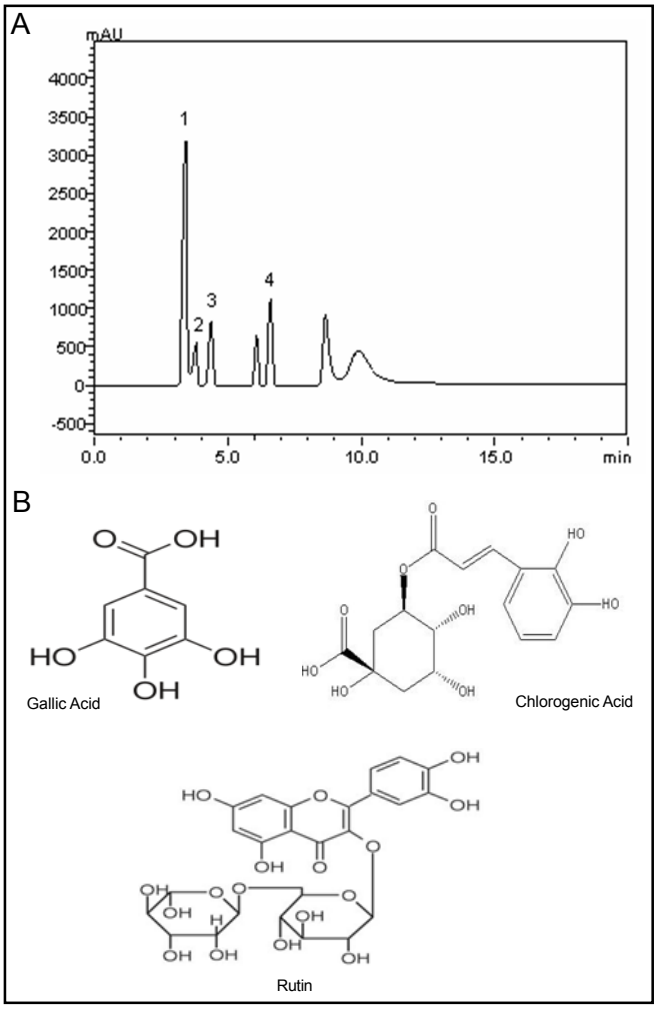

Fig. 1. A. HPLC fingerprint of the Syzygium cumini aqueous leaves extract ; A) showing typical patterns of gallic acid (1), kaempferol(2), chlorogenic acid (3) and rutin (4) in the 257 nm. B. Molecular structures of compounds found in the ASc.

added to the incubation medium at the concentrations cited above during the experimental procedure for 60 min. The activities of total ADA in the presence and absence of EHNA were measured. Platelets were treated with 100 µM EHNA, a potent ADA1 inhibitor. ADA1 activities were calculated by subtracting the activity of ADA2 (measured in the presence of EHNA) from that of total ADA. The combinations of EHNA plus ASc (100 and 200 µg/mL) were tested in platelets to better evaluate the inhibition of EHNA alone and EHNA plus ASc on ADA activities. The protein content used for the platelet experiment was adjusted to between 0.7 and 0.9 mg/mL. Results were expressed in units per liter (U/L).

TBARS levels

The samples of platelet suspensions were incubated with ASc at 100 µg/mL and 200 µg/mL concentrations for 15 min. Then, they were mixed with an equal volume of 15% (w/v) cold trichloroacetic acid in 0.25 M HCl and with an equal volume of 0.37% (w/v) thiobarbituric acid in 0.25 M HCl. Next, the samples were immersed in a boiling water bath for 10 min. After cooling and centrifugation, absorbance at 535 nm was measured, and results were expressed as nanomoles of MDA/mg protein [16].

CAT assay

CAT activity was measured by the method of Aebi [49]. 20 µL of platelets sample was added to a cuvette and the reaction was started by the addition of freshly prepared 0,5 M H2O2 in phosphate buffer (50mM, pH 7.0). The rate of hydrogen peroxide (H_2O_2) decomposition was measured spectrophotometrically at 240nm during 120 seconds. The activity of catalase was expressed as µmol H_2O_2/min/ ml platelets.

SOD assay

SOD (E.C.1.15.1.1) activity in tests was assayed spectrophotometrically as described by McCord and Fridowich [50]. This method is based on the capacity of SOD to inhibit autoxidation of adrenaline to adrenochrome. The color reaction was measured at 480nm. One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of epinephrine autoxidation by 50% at 26°C.

Protein determination

Protein content was determined according to Bradford [51], using bovine serum albumin as standard.

Statistical analysis

Statistical differences among groups were analyzed by Student's t-test and one-way analysis of variance, followed by Tukey's multiple range test, when appropriate. The correlations were assessed by Pearson rank correlation coefficient. Differences were considered statistically significant when $p<0.05$.

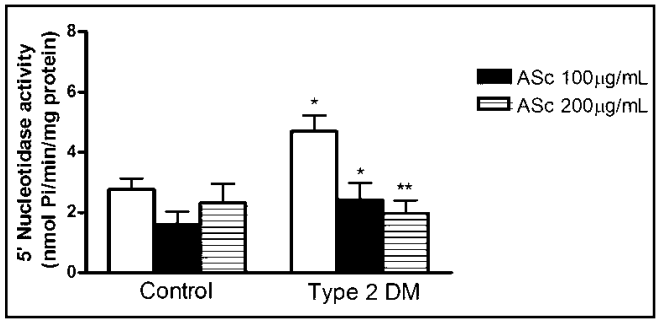

Fig. 2. *In vitro* exposure of platelets obtained from diabetics and control patients to ASc on 5'NT activity. All samples were run in triplicate. Data are reported as mean ± SEM and expressed as nmol Pi/min/mg protein. Statistically significant differences from controls and type 2 DM group as determined by ANOVA followed by Tukey multiple comparison test ($^{*}p<0.05$ compared with control group; $^{*}p<0.05$; $^{**}p<0.01$ compared with type 2 DM group).

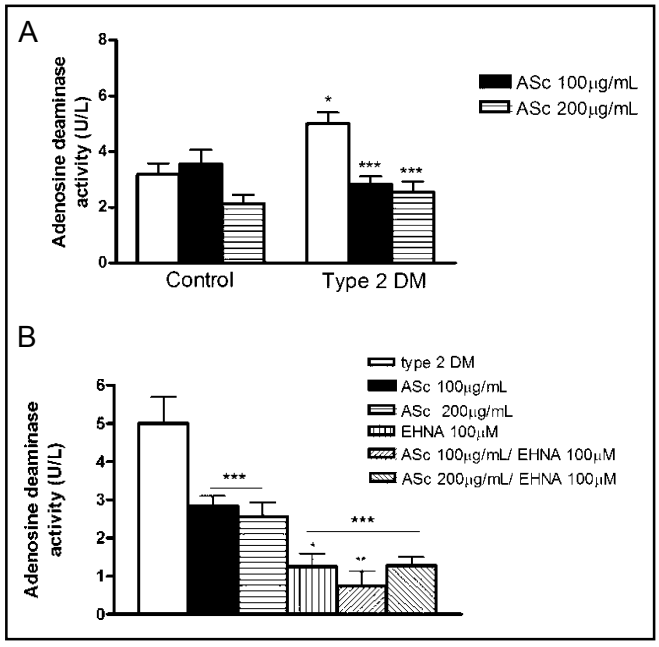

Fig. 3. A. Effect of ASc on ADA activity in platelets obtained from diabetics and control patients *in vitro*. All samples were run in triplicate. Data are reported as mean ± SEM and expressed as U/L. Statistically significant differences from controls and type 2 DM group, as determined by ANOVA followed by Tukey multiple comparison test ($^{*}p<0.05$ compared with control group; $^{***}p<0.001$ compared with type 2 DM group). B. Effect of *in vitro* exposure of platelets obtained from type 2 DM patients to ASc and 100 µM EHNA on ADA activity. All samples were run in triplicate. Data are reported as mean ± SEM and expressed as U/L. Statistically significant differences from controls and type 2 DM group, as determined by ANOVA followed by Tukey multiple comparison test ($^{*}p<0.05$ compared with ASc 100 µg/mL and 200 µg/mL; $^{**}p<0.01$ compared with ASc 100 µg/mL;$^{***}p<0.001$ compared with type 2 DM group).

Results

Biochemical and hematological characteristics of experimental groups

Biochemical and hematological parameters of the study subjects, type 2 DM and healthy controls are depicted in Table 1. Plasma glucose, serum triglyceride levels, and HbA1C levels in the group of diabetic patients were higher than in the healthy group (85.00%, 97.2%, and 34.78% respectively). Total cholesterol, HDL-C, creatinine, hemoglobin, hematocrit and platelets count of type 2 DM patients did not differ significantly from values in healthy subjects.

Chemical characterization of the extract

HPLC fingerprinting of the aqueous leaf extract showed an elution diagram when the peaks were grouped into three regions based on the UV absorption profile. These regions showed typical patterns of UV absorption, supporting the presence of gallic acid (1), kaempferol (2), chlorogenic acid (3) and rutin (4) in ASc (Fig. 1A), in the concentrations of 0,729% (1), 0,0093%(3) and 0,0304%(4), respectively. Molecular structures are shown in Fig. 1B. Therefore, HPLC analysis revealed that hydrolyzable tannins (galic), flavonoids (rutin) and phenolic are the major components of the extract. The results are similar to other studies [52-57].

Effect of ASc on 5'NT activity in platelets of type 2 DM subjects and healthy controls

5'NT activity in platelets of type 2 DM subjects were significantly higher than [mean 4.7 (±0.52)] that observed in the control group [mean 2.77 (±0.37), $p< 0.05$]. When platelets of diabetic patients were incubated with ASc (100 and 200 µg/mL) we observed 51.28% and 41.91% of 5'NT activity, respectively (Fig. 2). In the control group, however, no significant difference was found. We also observed a significant positive correlation between 5'NT activity and triglycerides levels of diabetic patients, as shown in Fig. 5A.

Effect of ASc on ADA activity in platelets of type 2 DM subjects and healthy controls

As illustrated in Fig. 3A, ADA activity in platelets of diabetic subjects were significantly higher than [mean 5.00 U/L (±0.4)] observed in controls [mean 3.18 (±0.4) U/L, $p< 0.05$]. Also, we evaluated the effects of ASc on the ADA activity. Experimental data demonstrated that ADA activity in platelets of diabetic subjects in the presence of

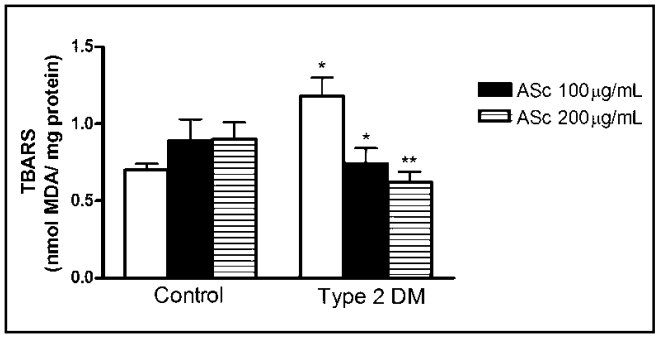

Fig. 4. Effect of *in vitro* exposure of platelets obtained from type 2 DM and control patients to ASc on TBARS levels. Data are reported as mean ± SEM. Statistically significant differences from controls and type 2 DM group as determined by ANOVA followed by Tukey multiple comparison test (* $p<0.05$ compared with control group; * $p<0.05$; ** $p< 0.01$ compared with type 2 DM group).

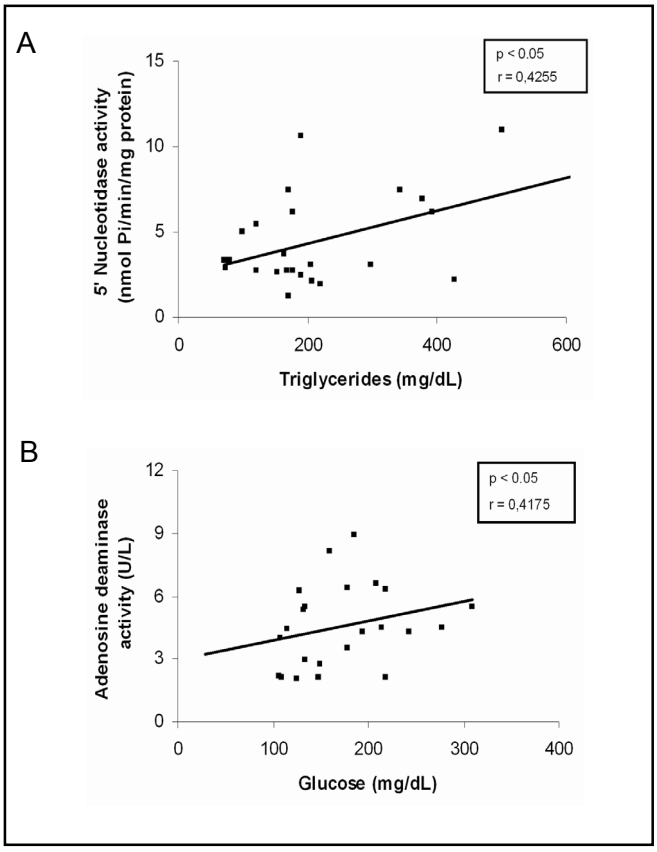

Fig. 5. A. Association between platelets 5'NT activities and triglycerides levels. There was observed a significant positive correlation ($r = 0,4255$, $p<0.05$). 5'NT activities are expressed in nmol Pi/min/mg protein, and triglycerides levels are expressed in mg/dL. B. Association between platelets ADA activities and glucose levels. There was observed a significant positive correlation ($r = 0,4175$, $p< 0.05$). ADA activities are expressed in U/L, and glucose levels are expressed in mg/dL.

100 and 200 µg/mL of ASc was 57.7% and 52.03%, respectively. However, in the control group ASc did not present any effect. When ASc (100 and 200 µg/mL) was incubated with EHNA we observed 15.03 % and 26.015% of ADA2 activity. These findings indicate that in the presence of ASc (100 and 200 µg/mL), ADA1 activity was 42.66 % and 26.015 % in platelets of type 2 DM subjects (Fig. 3B). We also observed a significant positive correlation between ADA activities and blood glucose levels, as shown in Fig. 5B.

TBARS levels and antioxidant enzymes

We observed that there was a significant increase in TBARS levels in platelets of type 2 DM subjects (1.18 ± 0.12) when compared to the control group [(0.7 ± 0.04), $p<0.05$] (Fig. 4). However, after the samples were incubated with ASc, we verified a significant reduction in TBARS levels to 0.74 (±0.10) and 0.62 (±0.07) at 100 µg/mL and 200 µg/mL, respectively, in diabetic subjects. As shown in Table 2, no significant difference was found between the SOD and CAT activities in the subjects studied.

	Control	type 2 DM
SOD (U SOD/ ml platelets)	97.46 ±3.17	95 .04 ± 4.01
CATALASE (umol H2O2/min/ml platelets)	146.2 ± 18.52	123.9 ± 12.44

Table 2. Levels of antioxidants enzymes in platelets of different study groups: SOD and CAT activities in the platelets of type 2 DM subjects and control group (data are expressed as mean ± SEM).There were no significant differences, as determined by Student's t-test.

Discussion

Many pathological alterations, such as vascular disease, increased platelet reactivity, and altered platelet morphology have been reported in patients with DM [58-60]. Platelets are an important source of purine signaling molecules for blood, such as ATP and ADP, which are related to several physiological events. Of particular interest, the metabolism of extracellular nucleotides plays an important regulatory role in the control of adequate hemostasis, mainly by regulating platelet coagulant status [61]. In the present study, we observed an increase of 5'NT activity in diabetic platelets (Fig. 2) would lead to an increase in the level of Ado. In fact, Ado, is an

important modulator of vascular tone, and it is a well-known inhibitor of platelet aggregation both *in vitro* and *in vivo* [62-64]. Recent studies have indicated a significant increase in 5'NT activities in platelets from diabetic type 2, hypertensive and diabetic type 2/hypertensive patients [35] and in platelets of Alloxan-treated rats [65].

Likewise, ADA activity was increased in platelets of diabetics (Fig. 3A). Corroborating with these results, Rutkiewicz and Górski [24] also found a significant elevation in ADA activity in tissues of diabetic rats induced with Streptozotocin (STZ), leading reduction Ado levels. Ado acts directly to stimulate or increase sensitivity to insulin [66] and to induce vasodilatation. Studies have suggested that the insulin is involved in the regulation of ADA activity in diabetes, and insulin administration is capable of decreasing the elevated activity of this enzyme in these tissues [67, 68]. Consequently, these effects would affect the development of vascular complications observed in the diabetic state, since Ado has an important role in preventing the thrombotic process. In fact, platelet activation, shape change, alterations in platelet cell membrane are implicated in different cellular signaling pathways in diabetic state [9].

We observed that ASc decreased significantly the 5'NT and ADA activities (Fig. 2 and Fig. 3A) in the platelets of subjects with type 2 DM. Based on our findings, we may suggest that ASc is able to maintain a level of Ado in the extracellular environment, which promotes vasodilatation and has an important protective role under pathophysiological conditions caused by the hyperglycemic state. Furthermore, studies showed that the thrombosis is inhibited by polyphenolic compounds which are present in ASc. These compounds can act through mechanisms like platelet activation associated to signal transduction and attenuation of generation of reactive oxygen species [69]. In addition, the inhibition of ADA can have protective effects on the ischemic tissues by preventing free radical-mediated injury and this is related to the improvement of cardiovascular activity [70]. In this regard, we can suggest that ASc may help in maintaining vascular integrity important in the hyperglycemic state. Further studies in the understanding of effect of ASc are currently under investigation by our group.

The diabetic state increased the production of levels of thiobarbituric acid in the circulation (Fig. 4). The hyperglycaemia can generate oxidative stress which has been suggested to play a primary role which may mediate tissue injury in Diabetes [71, 72]. In DM free radical over-production is not only specifically involved to the development of the complications [73], but is also involved in generating endothelial dysfunction and is linked to an increased risk of cardiovascular disease [74]. Not only in resting blood platelets but also during platelet activation that different reactive oxygen species are generated [10, 12].

Similarly, ASc reduced significantly TBARS levels (Fig. 4) in diabetic subjects. The main compounds present in the ASc (gallic acid, chlorogenic acid and rutin) can be responsible for these effects, since they have been known by their antioxidant capacity. The protection effect of ASc observed in the present study is consistent with the beneficial results found by Stanley [75], Moresco [76] and Bopp [44].

Controversies about activities of SOD and CAT have been reported in diabetics [77-79]. However, no significant difference was found in our study (Table 2). These controversial results may be due to a compensation mechanism of the body. Moreover, Hartnett [80] found a strong association between reduced SOD activity and diabetes, and considered their results to be unexpected in a disease with elevated oxidative compounds. Interestingly, we observed a moderate positive correlation between ADA activity and glucose levels (Fig. 5B) in diabetic subjects. Studies have been shown that a decrease in the level of Ado in circulation may be associated with a decrease in insulin-stimulated glucose uptake [81, 82]. Adenosine directly stimulates insulin activity via several processes, such as glucose transport, lipid synthesis, pyruvate dehydrogenase activity, leucine oxidation and cyclic nucleotide phosphodiesterase activity [24, 83, 84]. Then, the increase in ADA activity may reflect the decrease in adenosine circulation, leading the declining of sensivity by insulin and consequently the increase of glucose.

Also, we observed a positive correlation between triglycerides levels and 5'NT activity (Fig 5A). The level of serum lipids is usually raised in diabetes and seems to be associated with an increase in the risk of vascular disease. Significant disorders of lipid metabolism occur in the course of diabetes, manifested by total cholesterol level increase and changes in proportions and levels of serum lipid fractions [85]. There is evidence that adenosine can regulate several aspects of adipose tissue function including lipolysis [86, 87]. It is also pertinent that insulin administration *in vivo* decreases blood flow in rat white adipose tissue, and it has been hypothesized that this is achieved by decreased production of adenosine [88]. Furthermore, ADA1 activity was higher in platelets of type 2 DM subjects, corroborating with our previous

findings observed in erythrocytes of hyperglycemic samples [44].

In conclusion, ADA and 5'NT activities were higher in platelets of diabetics than control subjects, as well as TBARS levels. These oxidative phenomena were prevented by co-incubation with ASc. As a further confirmation of our hypothesis, in this paper we show that hyperglycemia is the prominent cause for the production of free radicals and modulation of enzymatics activities and platelet homeostasis, since these enzymes have a substantial function in the regulation of platelet aggregation. Moreover, the effects of ASc may be associated with the presence of gallic acid, chlorogenic acid and rutin. Thus, we can suggest that ASc could promote a compensatory response in platelet function, once that maintains levels of adenosine and contributes to the vasodilatation and inhibition of platelet aggregation beyond present antioxidant properties that play an important role in the prevention of various cardiovascular diseases.

Acknowledgments

The authors wish to thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). Karine is scholar of CAPES (Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior). We wish to thank the Programa Especial de Incentivo às Publicaçoes Internacionais (PRPGP/UFSM) and the Centro de Diagnóstico e Apoio Secundário (CEDAS).

References

1 Zimmet PZ, Mccarty DJ, De Couten MP: The global epidemiology of non insulin dependent diabetes mellitus and the metabolic syndrome. J Diabetes Complications 1997;11:60-68.

2 Dobrin JS, Lebeche D: Diabetic cardiomyopathy: signaling defects and therapeutic approaches. Expert Rev Cardiovasc Ther 2010;8:373-391.

3 Roffi M, Chew DP, Mukherjee D, Bhatt DL, White JA, Heeschen C, Hamm CW, Moliterno DJ, Califf RM, White HD, Kleiman NS, Théroux P, Topol EJ: Platelet glycoprotein IIb/IIIa inhibitors reduce mortality in diabetic patients with non-ST-segment-elevation acute coronary syndromes. Circulation 2001;104:2767-2771.

4 Wild S, Roglic G, Green A, Sicree R, King H: Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 2004;27:1047–1053.

5 Mathewkutty S, McGuire DK: Platelet perturbations in diabetes: implications for cardiovascular disease risk and treatment. Expert Rev Cardiovasc Ther 2009;7:541-549.

6 Natarajan A, Marshall SM, Worthley SG, Badimon JJ, Zaman AG: The presence of coronary artery disease increases platelet-dependent thrombosis in patients with type 2 diabetes mellitus. J Thromb Haemost 2008;6:2210-2213.

7 Marcus AJ, Broekman MJ, Drosopoulos JHF, Islam N, Pinsky DJ, Sesti C: Metabolic control of excessive extracellular nucleotide accumulation by CD39/ectonucleotidase- 1: implications for ischemic vascular diseases. J Pharmacol Exp Ther 2003;305:9-16.

8 Sprague RS, Stephenson AH, Bowles EA, Stumpf MS, Lonigro AJ: Reduced expression of G(i) in erythrocytes of humans with type 2 diabetes is associated with impairment of both cAMP generation and ATP release. Diabetes 2006;55:3588-3593.

9 El Haouari M, Rosado JA: Platelet signalling abnormalities in patients with type 2 diabetes mellitus: a review. Blood Cells Mol Dis 2008;41:119-23.

10 Iuliano L, Colavita AR, Leo R, Praticò D, Violi F: Oxygen free radicals and platelet activation. Free Radic Biol Med 1997;22:999-1006.

11 Pignatelli P, Pulcinelli FM, Lenti L, Gazzaniga PP, Violi F: Hydrogen peroxide is involved in collagen-induced platelet activation. Blood 1998;91:484-490.

12 Wachowicz B, Olas B, Zbikowska HM, Buczyñski A: Generation of reactive oxygen species in blood platelets. Platelets 2002;13:175-182.

13 Halliwell B: Antioxidants and human disease: a general introduction. Nutr Rev 1997;55:44-52.

14 Matkovics A: An overview of free radical research. Acta Biol Szegediensis 2003;47:93-99.

15 Tiwari AK: Antioxidants: new generation therapeutic base for treatment of polygenic disorders. Curr Sci 2004;86:1092-1102.

16 Olas B, Saluk-Juszczak J, Wachowicz B: D-glucaro 1,4-lactone and resveratrol as antioxidants in blood platelets. Cell Biol Toxicol 2008;24:189-199.

17 Olas B, Wachowicz B: Role of reactive nitrogen species in blood platelet functions. Platelets 2007;18:555-65.

18 Jahn B, Hansch GM: Oxygen radical generation in human platelets: dependence on 12-lipoxygenase activity and on the glutathione cycle. Int Arch Allergy Appl Immunol 1990;93:73-79.

19 Fürstenau CR, Trentin DdaS, Barreto-Chaves ML, Sarkis JJ: Ecto-nucleotide pyrophosphatase/phosphodiesterase as part of a multiple system for nucleotide hydrolysis by platelets from rats: kinetic characterization and biochemical properties. Platelets 2006;17:84-91.

20 Yegutkin GG: Nucleotide- and nucleoside-converting ectoenzymes: important modulators of purinergic signalling cascade. Biochim Biophys Acta 2008;1783:673-694.

21 Schmatz R, Schetinger MR, Spanevello RM, Mazzanti CM, Stefanello N, Maldonado PA, Gutierres J, Corrêa Mde C, Girotto E, Moretto MB, Morsch VM: Effects of resveratrol on nucleotide degrading enzymes in streptozotocin-induced diabetic rats. Life Sci 2009;84:345-350.
22 Ratech H, Martiniuk F, Borer WZ, Rappaport H: Differential expression of adenosine deaminase isozymes in acute leukemia. Blood 1988;72:1627-1632.
23 Ungerer JP, Oosthuizen HM, Bissbort SH, Vermaak WJ: Serum adenosine deaminase: isoenzymes and diagnostic application. Clin Chem 1992;38:1322-1326.
24 Rutkiewicz J, Górski J: On the role of insulin in regulation of adenosine deaminase activity in rat tissues. FEBS Letters 1990;271:79-80.
25 Conlon BA, Law W R: Macrophages are a source of extracellular adenosine deaminase-2 during inflammatory responses. Clin Exp Immunol 2004;138:14-20.
26 Desrosiers MD, Cembrola KM, Fakir MJ, Stephens LA, Jama FM, Shameli A, Mehal WZ, Santamaria P, Shi Y: Adenosine deamination sustains dendritic cell activation in inflammation. J Immunol 2007;179:1884-1892.
27 Bellé LP, De Bona KS, Abdalla FH, Pimentel VC, Pigatto AS, Moretto MB: Comparative evaluation of adenosine deaminase activity in cerebral cortex and hippocampus of young and adult rats: effect of garlic extract (Allium sativum L.) on their susceptibility to heavy metal exposure. Basic Clin Pharmacol Toxicol 2009;104:408-413.
28 Pimentel VC, Bellé LP, Pinheiro FV, De Bona KS, Da Luz SC, Moretto MB: Adenosine deaminase activity, lipid peroxidation and astrocyte responses in the cerebral cortex of rats after neonatal hypoxia ischemia. Int J Dev Neurosci 2009;27:857-62.
29 Pinheiro FV, Pimentel VC, De Bona KS, Scola G, Salvador M, Funchal C, Moretto MB: Decrease of adenosine deaminase activity and increase of the lipid peroxidation after acute methotrexate treatment in young rats: protective effects of grape seed extract. Cell Biochem Funct 2010;28:89-94.
30 Thomé GR, Mazzanti CM, Ahmed M, Corrêa M, Spanevello RM, Maldonado PA, Luchese C, Cargnelutti D, Morsch VM, Duarte MM, Fiorenza AM, Nogueira CW, De Bona KS, Moretto MB, Da Luz SC, Mazzanti A, Schetinger MR: Activity of ectonucleotidases and adenosine deaminase in rats exposed to cigarette smoke. Inhal Toxicol 2009;21:906-912.
31 Spanevello RM, Mazzanti CM, Bagatini M, Correa M, Schmatz R, Stefanello N, Thomé G, Morsch VM, Becker L, Bellé L, de Oliveira L, Schetinger MR: Activities of the enzymes that hydrolyze adenine nucleotides in platelets from multiple sclerosis patients. J Neurol 2010;257:24-30.
32 Spanevello RM, Mazzanti CM, Schmatz R, Thomé G, Bagatini M, Correa M, Rosa C, Stefanello N, Bellé LP, Moretto MB, Oliveira L, Morsch VM, Schetinger MR: The activity and expression of NTPDase is altered in lymphocytes of multiple sclerosis patients. Clin Chim Acta 2010;411:210-4.
33 Pilla C, Emanuelli T, Frasetto SS, Battastini AMO, Dias RD, Sarkis JJF: ATP diphosphoydrolase activity (apyrase, EC 3.6.1.5) in human blood platelets. Platelets 1996;7:225-230.
34 Frasetto SS, Schetinger MRC, Schierholt R, Webber A, Bonan CD, Wyse AT, Dias RD, Netto CA, Sarkis JJ: Brain ischemia alters platelet ATP diphosphohydrolase and 5'- nucleotidase activities in naive and preconditioned rats. Braz J Med Biol Res 2000;33:1369-1377.
35 Lunkes GI, Lunkes D, Stefanello F, Morsch A, Morsch VM, Mazzanti CM, Schetinger MR: Enzymes that hydrolyze adenine nucleotides in diabetes and associated pathologies. Thromb Res 2003;109:189-194.
36 Miron VR, Bauernmann L, Morsch AL, Zanin RF, Corrêa M, da Silva AC, Mazzanti C, Morsch VM, Lunkes GI, Schetinger MR: Enhanced NTPDase and 5'- nucleotidase activities in diabetes mellitus and iron-overload model. Mol Cell Biochem 2007;298:101-107.
37 Kitakaze M, Hori M, Sato H, Takashima S, Inoue M, Kitabatake A, Kamada T: Endogenous adenosine inhibits platelet aggregation during myocardial ischemia in dogs. Circ Res 1991;69:1402-1408.
38 Kawashima Y, Nagasawa T, Ninomiya H: Contribution of ecto-5'-nucleotidase to the inhibition of platelet aggregation by human endothelial cells. Blood 2000;96:2157-2162.
39 Sharma SB, Nasir A, Prabhu KM, Murthy PS: Antihyperglycemic effect of the fruit-pulp of Eugenia jambolana in experimental diabetes mellitus. J Ethnopharmacol 2006;104:367-373.
40 Teixeira CC, Fuchs FD: The efficacy of herbal medicines in clinical models: the case of jambolan. J Ethnopharmacol 2006;108:16-19.
41 Teixeira CC, Weinert LS, Barbosa DC, Ricken C, Esteves JF, Fuchs FD: Syzygium cumini (L.) Skeels in the treatment of type 2 diabetes: results of a randomized, double-blind, double-dummy, controlled trial. Diabetes Care 2004;27:3019-20.
42 Pandey M, Khan A: Hypoglycaemic effect of defatted seeds and water soluble fibre from the seeds of Syzygium cumini (Linn.) skeels in alloxan diabetic rats. Indian J Exp Biol 2002;40:1178-1182.
43 Ushida Y, Matsui T, Tanaka M, Matsumoto K, Hosoyama H, Mitomi A, Sagesaka Y, Kakuda T: Endothelium-dependent vasorelaxation effect of rutin-free tartary buckwheat extract in isolated rat thoracic aorta. J Nutr Biochem 2008;19:700-707.
44 Bopp A, De Bona KS, Bellé LP, Moresco RN, Moretto MB: Syzygium cumini inhibits adenosine activity and reduces glucose levels in hyperglycemic patients. Fundam Clin Pharmacol 2009;23:501-507.
45 Eidi A, Eidi M, Esmaeili E: Antidiabetic effect of garlic (Allium sativum L.) in normal and streptozotocin-induced diabetic rats. Phytomedicine 2006;13:624-629.
46 Zu Y, Li C, Fu Y, Zhao C: Simultaneous determination of catechin, rutin, quercetin, kaempferol and isorhamnetin in the extract of sea buckthorn (Hippophae rhamnoides L.) leaves by RP-HPLC with DAD. J Pharm Biomed Anal 2006;41:714-719.
47 Chan K, Delfert D, Junger KD: A direct colorimetric assay for Ca2+-ATPase activity. Anal Biochem 1986;157:375-378.
48 Giusti G, Gakis C: Temperature conversion factors, activation energy, relative substrate specificity and optimum pH of adenosine deaminase from human serum and tissues. Enzyme 1971;12:417-425.
49 Aebi H: Catalase in vitro. Methods Enzymol 1984;105:121-127.
50 McCord J, Fridowich I: Superoxide Dismutase, an enzymic function for erythrocuprein (hemocuprein). J Biol Chem 1969;244:6049-6055.
51 Bradford M: A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 1976;72:248-254.
52 Alberton JR, Ribeiro A, Sacramento LVS, Franco SL: Caracterização farmacognóstica do jambolão (Syzygium cumini (L.) Skeels). Rev Bras Farmacogn 2001;11:37-50.
53 Mahmoud II, Marzouk MAS, Maharram FA, El-Gindi MR, Hassan AMK: Acylated fl avonol glycosides from Eugenia jambolana leaves. Phytochemistry 2001;58:1239-1244.
54 Damasceno L, Ventura R, Cardoso J, Segura J: Diagnostic evidence for the presence of beta-agonists using two consecutive derivatization procedures and gas chromatography-mass spectrometric analysis. J Chromatogr B Analyt Technol Biomed Life Sci 2002;780:61-71.

55 Timbola AK, Szpoganicz AB, Monache FD, Pizzolatti MG: A new flavonol from leaves of Eugenia jambolana. Fitoterapia 2002;73:174-176.
56 Migliato KF, Baby AR, Zague V, Velasco MVR, Corrêa MA, Sacramento LVS, Salgado HRN: Ação farmacológica de Syzygium cumini (L.) Skeels. Acta Farm Bonaerense 2006;25:310-304.
57 Sharma SB, Nasir A, Prabhu KM, Murthy PS, Dev G: Hypoglycaemic and hypolipidemic effect of ethanolic extract of seeds of Eugenia jambolana in alloxan-induced diabetic rabbits. J Ethnopharmacol 2003;85:201-206.
58 Srivastava S, Joshi CS, Sethi PP, Agrawal AK, Srivastava SK, Seth PK: Altered platelet functions in non-insulindependent diabetes mellitus (NIDDM). Thromb Res 1994;76:451-461.
59 Ouvinã SO, La Greca RD, Zanaro NL, Palmer L, Sassetti B: Endothelial dysfunction, nitric oxide and platelet activation in hypertensive and diabetic type II patients. Thromb Res 2001;102:107-114.
60 Hekimsoy Z, Payzin B, Ornek T, Kandogan G: Mean platelet volume in Type 2 diabetic patients. J Diabetes Complicat 2002;18:173-176.
61 Kas-Deelen AM, Bakker WW, Olinga P, Visser J, de Maar EF, van Son WJ, The TH, Harmsen MC: Cytomegalovirus infection increases the expression and activity of ecto-ATPase (CD39) and ecto-5'- nucleotidase (CD73) on endothelial cells. FEBS Lett 2001;491:21-25.
62 Soslau G, Youngprapakorn D: A possible dual physiological role of extracellular ATP in modulation of platelet aggregation. Biochim Biophys Acta 1997;1355:131-140.
63 Sun B, Le SN, Lin S, Fong M, Guertin M, Liu Y, Tandon NN, Yoshitake M, Kambayashi J: New mechanism of action for cilostazol: interplay between adenosine and cilostazol in inhibiting platelet activation. J Cardiovasc Pharmacol 2002;40:577-85.
64 Borowiec A, Lechward K, Tkacz-Stachowska K, Skladanowski AC: Adenosine as a metabolic regulator of tissue function: production of adenosine by cytoplasmic 5'-nucleotidases. Acta Biochimica Polonica 2006;53:269-278.
65 Lunkes GI, Lunkes D, Morsch VM, Mazzanti CM, Morsch A, Miron VR, Schetinger MR: NTPDase and 5'-nucleotidase activities in rats with alloxan-induced diabetes. Diabetes Res Clin Pract 2004;65:1-6.
66 Jamal Z, Saggerson ED: Enzymes involved in adenosine metabolism in rat white and brown adipocytes. Effects of streptozotocin-diabetes, hypothyroidism, age and sex differences. Biochem J 1987;245:881-886.
67 Kurtul N, Pence S, karsu E, Kocoglu H, Aksoy Y, Aksoy H: Adenosine deaminase activity in the serum of type 2 diabetic patients. Acta Medica 2004;47:33-35.
68 Prakash MS, Chennaiah S, Murthy YSR, Anjaiah E, Rao SA, Suresh C: Altered adenosine deaminase activity in type 2 diabetes mellitus. JIACM 2006;7:114-117.
69 Wright B, Moraes LA, Kemp CF, Mullen W, Crozier A, Lovegrove JA, Gibbins JM: Structural basis for the inhibition of collagen-stimulated platelet function by quercetin and structurally related flavonoids. Br J Pharmacol 2010;159: 1312-1325.
70 Xia Y, Khatchikian G, Zweier JL: Adenosine deaminase inhibition prevents free radical-mediated injury in the postischemic heart. J Biol Chem 1996;271:10096-100102.
71 Baynes JW: Role of oxidative stress in development of complications in diabetes. Diabetes 1991;40:405-412.
72 Pfaffly JR: Diabetic complications, hyperglycemia & free radicals. Free Radic Biol Med 2001;77:222.
73 Brownlee M: Biochemistry and molecular cell biology of diabetic complications. Nature 2001;414:813-820.
74 Pennathur S, Heinecke JW: Mechanisms for oxidative stress in diabetic cardiovascular disease. Antioxid Redox Signal 2007;9:955-969.
75 Stanley Mainzen Prince P, Kamalakkannan N, Menon PV: Syzygium cumini seed extracts reduce tissue damage in diabetic rat brain. J Ethnopharmacol 2003;84:205-209.
76 Moresco RN, Sperotto RL, Bernardi AS, Cardoso RF, Gomes P: Effect of the aqueous extract of Syzygium cumini on carbon tetrachloride-induced hepatotoxicity in rats. Phytother Res 2007;21:793-795.
77 Sundaran RK, Bhaskar A, Vijayalingam S, Vishwanathan M, Mohan R, Shanmugasundaram KR: Antioxidant status and lipid peroxidation in type II diabetes mellitus with and without complications. Clin Sci 1996;90:255-260.
78 Vijayalingam S, Parthiban A, Shanmugasundran KR, Mohan V: Abnormal antioxidant status in impaired glucose tolerance and non-insulin dependent diabetes mellitus. Diabet Med 1996;13:715-719.
79 Ayidin A, Orhan H, Sayal A, Ozata M, Sahir G, Isimer A: Abnormal antioxidant status in impaired glucose tolerance and non-insulin dependent diabetes mellitus.Clin Biochem 2001;34:65-70.
80 Hartnett ME, Stratton RD, Browne RW, Rosner BA, Lanham RJ, Armstrong D: Serum markers of oxidative stress and severity of diabetic retinopathy. Diabetes Care 2000;23:234-240.
81 Kuroda M, Honnor RC, Cushman SW, Londos C, Simpson IA: Regulation of insulin-stimulated glucose transport in the isolated rat adipocyte. J Biol Chem 1987;262:245-253.
82 Wyatt DA, Edmunds MC, Rubio R, Berne RM, Lasley RD, Mentzer RM Jr: Adenosine stimulates glycolytic flux in isolated perfused rat hearts by A1- adenosine receptors. Am J Physiol 1989;257:952-957.
83 McLane MP, Black PR, Law WR, Raymond RM: Adenosine reversal of in vivo hepatic responsiveness to insulin. Diabetes 1990;39:62-69.
84 Hoshino T, Yamada K, Masuoka K, Tsuboi I, Itoh K, Nonaka K,Oizumi, K: Elevated adenosine deaminase activity in the serum of patients with diabetes mellitus. Diabetes Res Clin Pract 1994;25:97-102.
85 Dunn FL: Management of dyslipidemia in people with type 2 diabetes mellitus. Rev Endocr Metab Disord 2010;11:41-51.
86 Schwabe U, Schönhöfer PS, Ebert R: Facilitation by adenosine of the action of insulin on the accumulation of adenosine 3':5'-monophosphate, lipolysis, and glucose oxidation in isolated fat cells. Eur J Biochem 1974;46:537-545.
87 Fredholm BB: Local regulation of lipolysis in adipose tissue by fatty acids, prostaglandins and adenosine. Med Biol 1978;56:249-261.
88 Madsen J, Malchow-Moller A: Effects of glucose, insulin and nicotinic acid on adipose tissue blood flow in rats. Acta Physiol Scand 1983;118:175-180.