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Original article

Cupuaçu extract reduces nitrosative stress and modulates inflammatory mediators in the kidneys of experimental diabetes

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SUMMARY

Background & aims: We have previously reported an increased nitrosative stress in the kidneys of diabetic animals, which was reduced by an antioxidant probiotic. The present study evaluated whether the extract of cupuaçu (EC), an antioxidant compound rich in polyphenols and theograndins, when administered at a dose that can be reasonably obtained through daily consumption, could delay the onset of diabetic complications in the kidney.

Methods: Mouse immortalized mesangial cells (MiMC) were placed in medium normal glucose (NG) or high glucose (HG), with or without EC (500, 100, 50 or 10 μg/mL) during 24, 48 or 72 h for analysis of viability, proliferation, nitric oxide (NO) levels and reactive oxygen species or nitrogen (ROS/RNS). Male, adult Wistar rats were distributed in 4 groups: control (CTL) and diabetic (DM) who received water; CTLEC and DMEC who received 1 mL/day of EC (1 g/mL), via gavage for 8 consecutive weeks. After, metabolic profile and renal function were evaluated and, kidneys were collected for analysis of NO, ROS, 3-nitrotyrosine (3-NT), endothelial nitric oxide synthase (eNOS), IL-6, IL-10, TNF-α and NF-κB p65.

Results: The MiMC showed normal viability in all groups, demonstrating that EC had no cytotoxic effect at doses on 24, 48 or 72 h. MiMC under HG presented significant increase in proliferation, NO and ROS vs. NG; these parameters were significantly reduced after 72 h of EC treatment ($P < 0.05$). DMEC showed a significant reduction of feed intake, ROS and NO, 3-NT, IL-6 and eNOS vs. DM ($P < 0.05$). Supplementation with EC at a dose consumed daily could improve control of nitrosative stress, combined with reduction of inflammatory factors, suggesting the importance of bioactive compounds as non-pharmacological adjuvant therapy to delay kidney complications in diabetic patients.

1. Introduction

Diabetes mellitus is a non-transmissible chronic disease, characterized by hyperglycemia, which favors the increase of ROS production, as hydrogen peroxide and superoxide radicals [1], via glucose auto-oxidation in various tissues. This process can result in high levels of oxidative/nitrosative stress with sequential reduction of NO bioavailability, because it could be sequestered by these species, especially superoxide anion, generating peroxynitrite; this in turn is a reactive nitrogen specie (RNS) able to change a variety of biomolecules, such as amino acids, proteins, enzymes and enzyme cofactors. Peroxynitrite can lead to nitration of tyrosine residues forming 3-NT, considered a nitrosative stress marker, which can cause structural and functional damage to the cells [2,3]. In this situation, it occurs the uncoupling of eNOS, which favors a higher production of ROS/RNS [4].

Hyperglycemia and oxidative/nitrosative stress contribute to microvascular damage, resulting in diabetic nephropathy (DN); this is characterized by the expansion of mesangial extracellular matrix (ECM) with consequent reduction in the glomerular filtration, affecting about 20–30% of the diabetic patients [5].

ROS/RNS are also responsible for NF-κB activation, this, in turn, elevates the expression of pro-inflammatory markers, as tumor

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necrosis factor-*alpha* (TNF-α) and interleukin-6 (IL-6), among others [6]. Previous studies of our group showed an increase of oxidative/nitrosative stress and inflammatory parameters in the renal tissue of diabetic animals; however, these markers were reduced after antioxidant treatment, accompanied by improvement in the NO bioavailability and renal function, delaying the progression of the DN [7–9].

The *Theobroma* genus is composed of 22 species, among them, the cocoa (*Theobroma cacao* L.), which has a certain similarity with cupuaçu (*Theobroma grandiflorum*), since both are part of the same genus; however, they have differentiated bioactive compounds; cocoa has important phenolic compounds such as proanthocyanidins and epicatechins, being associated with the reduction of lipoperoxidation and increased antioxidant capacity in humans, contributing to protection mechanisms in cardiovascular diseases. Cupuaçu has another type of flavonoids, as theograndins, which have been related to the reduction of oxidative stress in chronic diseases [10,11].

A comparative study between cocoa and cupuaçu showed that the latter had best results in the improvement of antioxidant profile and reduction of triglycerides in diabetic animals, although cupuaçu had a lower amount of phenolic compounds and palmitic fatty acid, suggesting the importance of the bioactive compounds contained in this product. Moreover, cupuaçu pulp is fiber-rich, which can be used in other products providing a differentiated more than other fruit pulps [12].

The objective of the present study was to assess the effects of cupuaçu extract on nitrosative stress and inflammatory mediators in diabetic kidneys of rats.

2. Material and methods

2.1. Assays in vitro

2.1.1. EC treatment in MiMC

The EC was kindly provided by Prof. Maria Ines Genovese, Department of Food and Experimental Nutrition, Faculty of Pharmaceutical Sciences, University of Sao Paulo, which was prepared as follows: frozen pulp of cupuaçu (100 g) acquired from "Sítio do Bello ME" (SP, Brazil) was diluted in 200 mL of distilled water and homogenized. Then, EC was filtered by 0.22 μm syringe and stored in sterile tubes in freezer at −20. To obtain final concentrations of 500, 100, 50 or 10 μg/mL used in the treatment, the EC was diluted in MiMC culture medium.

The MiMC SV40 MES 13 purchased from American Type Culture Collection (ATCC − CRL 1927) was provided by Nephrology Division − UNIFESP (SP, Brazil). The cells were grown and kept at 95% air and 5% CO_2 humidified environment at 37 °C in Dulbecco's modified Eagle's medium (DMEM) and F12 (3:1), containing 5% fetal bovine serum (FBS) and penicillin (50 U/mL)/streptomycin (50 mg/mL) (Life Technologies, SP, Brazil). The medium was replaced every 48 h. All the experiments were performed with cells between 10th and 20th passages. We used the dose of 30 mM as a high glucose concentration to mimics diabetes, as described in the literature [13].

At semi-confluence of approximately 50–60% (ideal cell density for avoid the over-grown, as previously tested, in our laboratory), the cells were incubated in FBS free medium by 24 h and after this period, the medium was replaced and the cells were treated during 24, 48 or 72 h with medium plus 0.5% FBS according to the experimental groups: normal glucose (NG), incubated in medium containing a standard concentration of 6.7 mM D-glucose; high glucose (HG), incubated with medium containing D-glucose at final concentration of 30 mM and the osmolarity control, which was incubated in medium supplemented with mannitol at final concentration of 30 mM.

2.1.2. Cell viability and proliferation of MiMC after EC treatment

The MiMC were incubated with 5% FBS in 96-well plates at concentration of 3×10^3 cells/mL per well. At semi-confluence (50–60%), the cells were exposed to FBS free medium by 24 h for synchronization. Then, the medium was replaced for DMEM, containing 0.5% FBS in conditions of NG, MA, HG or HG plus 500, 100, 50 or 10 μg/mL of EC for 24, 48 or 72 h. After this time, the cells were trypsinized and centrifuged, the supernatant was discarded and the cells were resuspended in 1 mL of PBS. Viability and cytotoxicity were assessed using trypan blue (0.4%) and the cells were counted utilizing the equipment Countess, an automatic cell counter (Life Technologies, USA). The proliferation was evaluated by methyl thiazole tetrazolium (MTT) assay (Sigma Chemical, MO, USA) assay. Optical density (OD) was analyzed with a microplate reader at 570 nm. The OD of the NG cells was calculated by relative value of 100. The results were obtained from the average of three independent experiments done in octuplicate wells.

2.1.3. Analysis of NO and ROS in MiMC after EC treatment

The 12-well culture plates with a concentration of 7×10^3 cells/mL per well were treated, according to their respective groups. After the treatment, the supernatant was collected and stored at −20 °C. NO levels in the supernatant were analyzed by chemiluminescent method between NO and ozone in the equipment Nitric Oxide Analyzer (Sievers Instruments Inc, USA), which is a sensitive NO detector in liquid samples and, the results were obtained from three independent experiments done in triplicate wells.

The 2.7 dichlorodihydrofluorescein (DCF), a fluorescent probe that reacts with intracellular hydrogen peroxide and peroxidases, was utilized to evaluate intracellular ROS levels. The MiMC were incubated with 9 μM of DCF-diacetate (DCFH-DA, Sigma Chemical, MO, USA) at 37 °C for 30 min, the medium was then removed and the cells were washed with PBS. The fluorescence was measured using a fluorescence plate reader (Synergy HT, Biotek, USA); excitation was read at 480 nm and emission was detected at 520 nm. The relative ROS production was expressed as the mean fluorescence intensity. The results were obtained from three independent assays done in octuplicate wells.

2.2. Assays in vivo

2.2.1. EC treatment in animals

For *in vivo* experiments, the frozen pulp of cupuaçu (200 g) obtained from "Sítio do Bello ME" (SP, Brazil), protected from light, was diluted in 200 mL of filtered water (1 g/mL), homogenized, sifted and aliquoted in sterile tubes, in the daily amount of use, avoiding repeated thawing of product, being stored in freezer at −20 until the moment of use. All these procedures were performed in dark environment to prevent oxidation of the compounds.

Male Wistar rats with 8 weeks and ±200 g were purchased from CEDEME (Central Animal Housing, UNIFESP, SP, Brazil). The animal protocol was approved by the Ethics Committee in Research at Federal University of Sao Paulo, under number 544396. The animals remained inside boxes in the Nephrology Division (UNIFESP, SP, Brazil) at a light–dark cycle of 12/12 h with temperature of 22 ± 1 °C. The rats had free access to standard chow (Nuvilab, PR, Brazil) and water. The rats were distributed into four groups, with n = 5 each: CTL (control); CTLEC (control treated with cupuaçu); DM (diabetic) and DMEC (diabetic treated with cupuaçu).

After few days of adaptation, 8-week-old rats received a single intravenous dose of 45 mg/kg streptozotocin (STZ, Sigma Chemical,

MO, USA) diluted in STZ-vehicle (cold citrate buffer, 0.1 M, pH 4.5). Control rats received STZ-vehicle. After period of 72 h of the induction, the glycemia was checked through glucometer in a blood sample, which was collected from the caudal vein. Diabetes was determined when fasting glycemia was found ≥200 mg/dL.

The CTLEC and DMEC received extract of cupuaçu (EC) beginning on the 4th day after induction of the diabetes. EC (1 mL/day) was given for 8 consecutive weeks, via gavage, in the concentration of 1 g/mL, which is equivalent to one cup (200 mL/day) of cupuaçu juice for a person, with no sugar added. The CTL and DM groups received EC-vehicle (water). The rats were allocated in individual metabolic cages after treatment, with free access of water and chow, to collect metabolic data and 24-h urine. Subsequently, the animals were fasted for a period of 3 h and, after this, the fasting glycemia was determined via glucometer in a blood sample, which was collected from the caudal vein; then the animals received anesthesia of ketamine hydrochloride (67 mg/kg) and xylazine hydrochloride (8 mg/kg) by intramuscular injection and blood was withdrawn from the retro-orbital plexus. The samples were stored at −20 °C. The animals were euthanized with high dose of anesthetic (90 mg/kg of ketamine hydrochloride and 18 mg/kg of xylazine hydrochloride at, intraperitoneally), followed by an incision of the diaphragm and the kidneys were removed.

2.2.2. Renal function assessment in animals after EC treatment

The Urea kit CE was utilized to estimate plasma and urinary urea concentrations; colorimetric assay Creatinine kit was used to measure creatinine; uric acid was analyzed by Acid Uric Liquiform kit and proteinuria was evaluated by Sensiprot kit. All kits were purchased from Labtest (MG, Brazil).

2.2.3. Estimation of nitrosative stress and inflammatory markers in animals after EC treatment

The superoxide anion level in the renal cortex was detected indirectly using nitroblue tetrazolium (NBT, Sigma, MO, USA) protocol and the optical density (OD) was read at 560 nm. NO was measured in renal cortex of the animals after 8 consecutive weeks of EC treatment using chemiluminescence method as previously described.

The protein content of 3-NT, eNOS, IL-10, NF-κB p65, actin (Santa Cruz Biotechnology, TX, USA), IL-6 and TNF-α (Abcam, Cambs, UK) was analyzed in the renal cortex samples, which were homogenized with K-HEPES buffer plus protease inhibitors (cocktail I, Millipore, MA, USA). The homogenate was centrifuged at 2000g, the protein was quantified by Bradford method and 50 μg of protein were used on polyacrylamide gel (10%), being transferred to a nitrocellulose membrane, which were probed against the respective secondary antibodies. The bands were visualized with a chemiluminescent substrate (immobilon, Millipore, MA, USA) in the equipment Alliance 4.7 Uvitec (Cambs, UK) and, the relative protein content was normalized using actin antibody.

2.3. Statistical analysis

The sample size was determined after analysis of previous studies performed in our laboratory. The data were shown on average and standard error media (SEM). First, it was utilized the Kolmogorov Smirnov normality test, followed by one-way analysis of variance (ANOVA) and Newman–Keuls Multiple Comparison post-test (parametric data) for cell proliferation (48 h), metabolic profile, renal function, ROS, NO renal, 3-NT and IL-10 or Kruskal–Wallis and Dunn's Multiple Comparison post-test (non-parametric data) for cell viability, cell proliferation (24 or 72 h), NO (24, 48 or 72 h), eNOS, IL-6, TNF-α and NF-κB p65. Values were considered statistically significant for $P < 0.05$ and tests were performed on the software GraphPad Prism 5.0.

3. Results

3.1. Assays in vitro

3.1.1. Viability and cell proliferation of MiMC after EC treatment

After treatment with EC, the viability of MiMC in NG medium at all doses (500, 100, 50 or 10 μg/mL) was above 95% after 24, 48 or 72 h, showing the non-cytotoxic effect of EC in MiMC (Fig. 1a).

In Fig. 1b, the cell proliferation (%) increased significantly in the HG group (130 ± 5; 189 ± 12; 342 ± 14) compared to NG, respectively 24, 48 or 72 h after EC. The addition of EC 50 or 10 μg/mL significantly reduced the proliferation of MiMC at 24 h (111 ± 6 and 118 ± 6) vs. HG; all studied doses of EC (500, 100, 50 or 10 μg/mL) significantly reduced the proliferation at 48 h (150 ± 9; 143 ± 8; 146 ± 7; 152 ± 8) or 72 h (218 ± 11; 217 ± 12; 237 ± 12; 223 ± 13) compared to HG, respectively.

3.1.2. NO and ROS generation in MiMC after EC treatment

The NO (nmol/mg) levels after 24 h of EC were significantly increased in HG (177 ± 12) when compared to NG group (111 ± 11). For the other hand, EC 100, 50 or 10 μg/mL (104 ± 10; 126 ± 19; 104 ± 10; respectively) compared to HG group, significantly reduced NO. After 48 or 72 h, HG was significantly increased (245 ± 20; 384 ± 51) compared to NG (133 ± 8; 145 ± 8), respectively, however, there was a significant reduction after all EC doses 500, 100, 50 or 10 μg/mL (137 ± 7; 120 ± 8; 132 ± 4; 137 ± 20), after 48 h and 72 h (167 ± 18; 149 ± 24; 170 ± 24; 165 ± 22), respectively, when compared to HG, as seen in Fig. 2a.

The ROS generation measured by the mean fluorescence intensity of DCF (units of fluorescence) showed a significant increase in the HG (3268 ± 120) compared to NG (1599 ± 170); EC 500 μg/mL was able to significantly reduce ROS after 72 h when compared to HG (Fig. 2b).

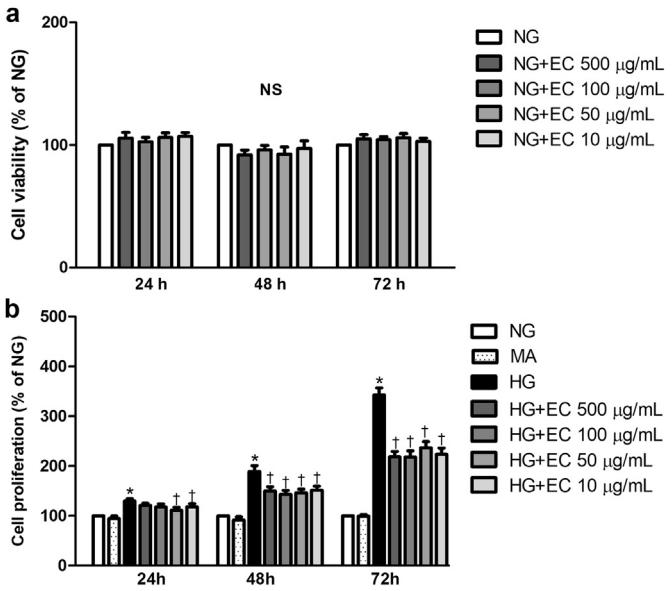

Fig. 1. EC treatment on cell viability (**a**) or proliferation (**b**) in MiMC, after 24, 48 or 72 h. EC: extract of cupuaçu. MiMC: mouse immortalized mesangial cells. NG: normal glucose (6.7 mM); MA: mannitol (30 mM); HG: high glucose (30 mM). Values were expressed on average and SEM. ANOVA with Newman–Keuls or Kruskal Wallis with Dunn's; NS: not significant; $P < 0.05$: *vs. NG; †vs. HG.

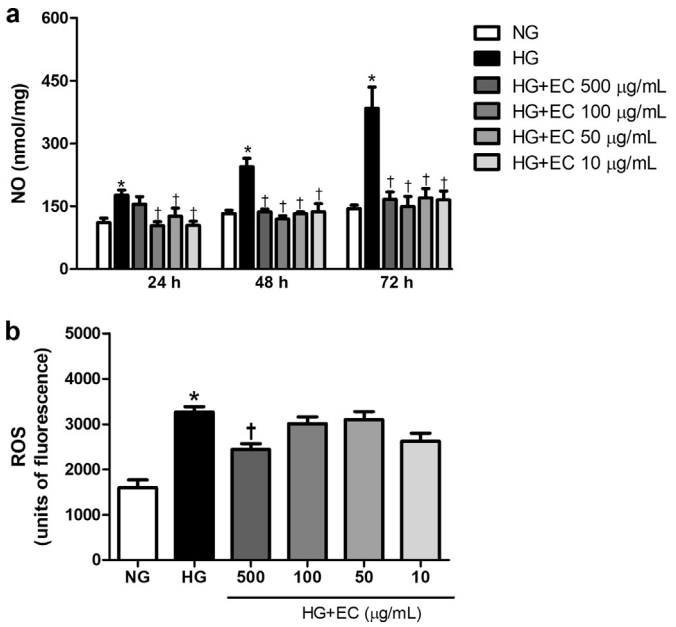

Fig. 2. NO (**a**) or ROS (**b**) generation after EC treatment in MiMC. EC: extract of cupuaçu; NO: nitric oxide; ROS: reactive oxygen species; MiMC: mouse immortalized mesangial cells. NG: normal glucose (6.7 mM); HG: high glucose (30 mM). Values were expressed on average and SEM. ANOVA with Newman–Keuls or Kruskal Wallis with Dunn's; $P < 0.05$: *vs. NG; †vs. HG.

3.2. Assays in vivo

3.2.1. Metabolic data and renal function in animals after EC treatment

There was no statistical difference between the control groups (CTLEC vs. CTL). The diabetic vs. control groups revealed significant differences in: fasting blood glucose; water and chow intake; diuresis; weight gain; plasmatic and urinary of urea, creatinine and uric acid and proteinuria. However, in the DMEC group, just chow intake was significantly decreased compared to DM. Although not significant, there was a partial improvement in the glycemia, water intake, diuresis, urea and creatinine in DMEC vs. DM, as seen in Table 1.

3.2.2. Nitrosative stress in animals after EC treatment

There was a significant increase in the renal cortex NO levels (nmol/mg) in DM (15.2 ± 3.2) vs. CTL (7.9 ± 0.5). However, there was a significant decrease in this parameter in the DMEC (8.0 ± 1.6) compared to DM (Fig. 3a).

In relation to ROS production, demonstrated by the renal superoxide anion (OD), there was significant raise in the DM (0.30 ± 0.05) vs. CTL (0.16 ± 0.02); there was no difference between DMEC (0.21 ± 0.03) vs. CTLEC (0.19 ± 0.01), however, DMEC had a reduction of 30% vs. DM, although not significant, as seen in Fig. 3b.

The 3-NT in renal cortex was significantly increased in the groups DM (1.78 ± 0.12) vs. CTL (0.87 ± 0.11) and DMEC (1.24 ± 0.09) vs. CTLEC (0.76 ± 0.07); there was a significant reduction in DMEC vs. DM. On the other hand, the eNOS was significantly increased in DM (1.44 ± 0.20) vs. CTL (0.94 ± 0.04), without showing statistical difference between DMEC (0.94 ± 0.09) vs. CTLEC (0.94 ± 0.05); revealing a significant decrease in the DMEC vs. DM, as in Fig. 3C.

3.2.3. Inflammatory markers in animals after EC treatment

The IL-6 was significantly increased in DM (1.11 ± 0.05) vs. CTL (0.77 ± 0.10); DMEC (0.87 ± 0.04) was significantly reduced vs. DM and there was no significant difference between DMEC vs. CTLEC (0.79 ± 0.04). In relation to IL-10, there was a significant reduction in DM (0.77 ± 0.06) vs. CTL (1.17 ± 0.05) and DMEC (0.79 ± 0.04) vs. CTLEC (1.21 ± 0.09), as seen in Fig. 4a.

The TNF-α was significantly increased in DM (1.08 ± 0.08) vs. CTL (0.42 ± 0.03) and DMEC (1.00 ± 0.09) vs. CTLEC (0.47 ± 0.02) and, NF-κB p65 was significantly increased in DM (2.12 ± 0.22) vs. CTL (0.68 ± 0.02), without showing statistical difference between DMEC (1.14 ± 0.08) vs. CTLEC (0.65 ± 0.02); DMEC was significantly reduced vs. DM, as seen in Fig. 4b.

4. Discussion

For our knowledge, it is the first time that the control of nitrosative stress by EC was shown in the experimental diabetes. The EC in MiMC had no cytotoxic effect and when these cells were treated with high glucose plus EC, there was a reduction in the cell proliferation, ROS and NO levels. On the other hand, in diabetic rats induced by STZ, although the reduction in hyperglycemia was not statistically significant, it had an impact on the feed intake, reducing the polyphagia, and probably, combined with the modulation of nitrosative stress, it resulted in downregulation of NF-κB p65, regulating the inflammatory mediators.

Antioxidants such as cocoa flavanols have a great ability to eliminate free radicals, which is much higher in cocoa than in green tea, black tea or red wine [14]. Other study revealed that chemoprotective effect of cocoa flavanols had key role in diseases such

Table 1
Metabolic parameters and renal function in rats after EC.

Parameters	CTL		CTLEC		DM		DMEC	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Fasting glucose (mg/dL)	113.00	1.38	113.50	2.31	414.80	35.89*	371.00	15.72‡
Water intake (mL/day)	33.80	3.32	34.00	9.76	90.20	17.99*	76.20	14.78
Chow intake (g/day)	14.60	0.62	17.88	1.77	25.44	2.08*	19.38	2.64§
Diuresis (mL/day)	12.60	0.92	15.00	2.28	61.40	12.62*	49.00	11.53‡
Weight gain (%)	27.20	1.50	22.80	0.58	12.80	3.97*	14.00	4.90
Plasmatic urea (mg/dL)	19.15	1.66	23.82	1.79	43.17	5.47*	39.10	7.56‡
Plasmatic creatinine (mg/dL)	0.33	0.03	0.38	0.03	0.48	0.01*	0.38	0.01
Plasmatic uric acid (mg/dL)	0.38	0.03	0.28	0.07	0.71	0.06*	0.58	0.06‡
Urinary urea (mg/dL)	1702.00	182.90	1438.00	134.00	623.00	85.61*	857.90	250.90‡
Urinary creatinine (mg/dL)	110.00	8.00	87.61	11.00	39.80	14.65*	38.90	15.59‡
Urinary uric acid (mg/dL)	16.69	1.47	19.33	1.15	4.30	1.05*	4.39	0.69‡
Proteinuria (mg/dL)	13.33	1.07	14.24	1.21	20.07	1.70*	19.29	1.72‡

Values were expressed on average and SEM. ANOVA with Newman–Keuls. Control (CTL); control plus EC (CTLEC); diabetic (DM); diabetic plus EC (DMEC); n = 5 for all groups; $P < 0.05$: *vs. CTL; ‡vs. CTLEC; §vs. DM.

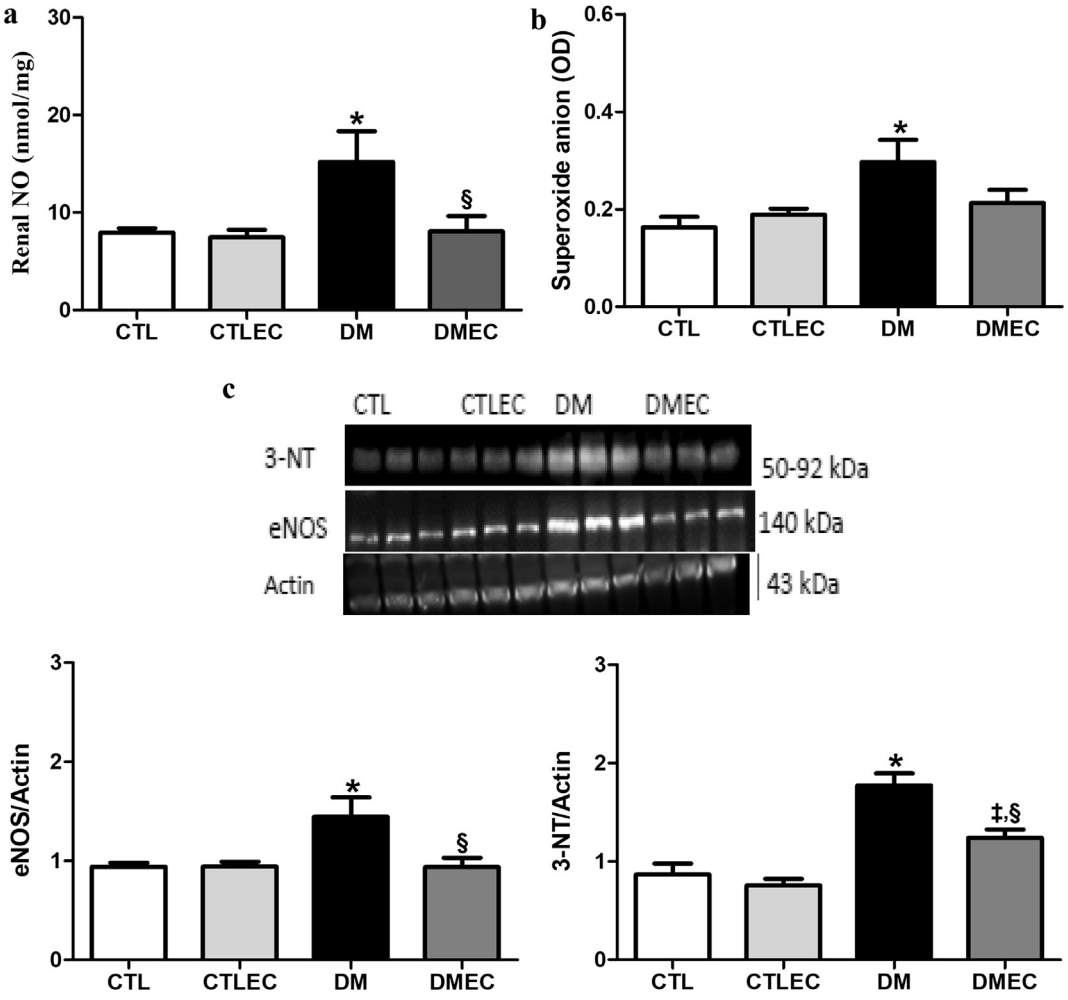

Fig. 3. NO (**a**), superoxide anion (**b**), 3-NT and eNOS (**c**) in the kidneys after EC treatment. NO: nitric oxide; 3-NT: 3-nitrotyrosine; eNOS: endothelial nitric oxide synthase; EC: extract of cupuaçu. Control (CTL); control plus EC (CTLEC); diabetic (DM); diabetic plus EC (DMEC); n = 4–5 per group. Values were expressed on average and SEM. ANOVA with Newman–Keuls or Kruskal Wallis with Dunn's; $P < 0.05$: *vs. CTL; ‡vs. CTLEC; §vs. DM.

as type 2 diabetes, in which there is excessive ROS production [15]. Cupuaçu has recently been studied due to its content of polyphenols and antioxidant capacity, with some similarity to cocoa, since both belong to the same gender *Theobroma*. However, they have differentiated bioactive compounds, according to a study that analyzed the action of both in diabetic animals, revealing that cupuaçu had greater antioxidant effect than cocoa [12]. Our study demonstrated that cupuaçu had important antioxidant action, since it was able to reduce oxidative and nitrosative stress in MiMC and in diabetic animals.

It is known in the literature that high glucose concentration induces cell proliferation and collagen production, via increased oxidative stress and TGF-β1 in mesangial cells [16,17]. These data corroborate our study, since the high concentration of glucose promoted cell proliferation, which was not seen in the mannitol group; besides, the elevated glucose increased NO and ROS levels in MiMC and diabetic kidney of animals. EC reduced these effects, suggesting the nitrosative stress contribution in MiMC proliferation under high glucose and in the regulation of inflammatory markers, as such as NF-κB p65 in diabetic animals.

In relation to type 1 diabetes model, our study is in agreement with the literature, since this STZ-induced diabetes model leads to the destruction of pancreatic β cells and impairment of insulin secretion, causing metabolic and physical abnormalities, such as reduced weight gain and classics symptoms of hyperglycemia (polyphagia, polydipsia and polyuria) [18]. In our study, there was a slight reduction in water intake and diuresis and a significant improve in the feed intake in DMEC vs. DM, which could be explained by the reduction of hyperglycemia [19].

Hyperglycemia leads to dysfunction of several organs mainly the kidneys, contributing to progression of DN, increasing morbidity and mortality in diabetic patients [20]. In our study, the kidneys of DM group were damaged in relation to CTL, and there was an improvement in the parameters of renal function after EC treatment. Hyperglycemia plays an important role in the pathogenesis of long-term complications, which is associated with the generation of ROS/RNS [21,22], being observed a high risk of renal lesions in diabetic patients with little glycemic control [23]. However, in our study, the changes observed in renal function after EC treatment, had no statistical significance, perhaps because time of this study was not enough to demonstrate a striking amelioration of renal function or the kidneys could be more protected from oxidative/nitrosative damage if the cupuaçu was given before the induction of diabetes.

The interaction of NO and superoxide is emerging as important regulator of renal function. Therefore, the focus had been on the pro-oxidant pathways of NO under normal and pathological conditions. Studies with animals and humans have attempted to find

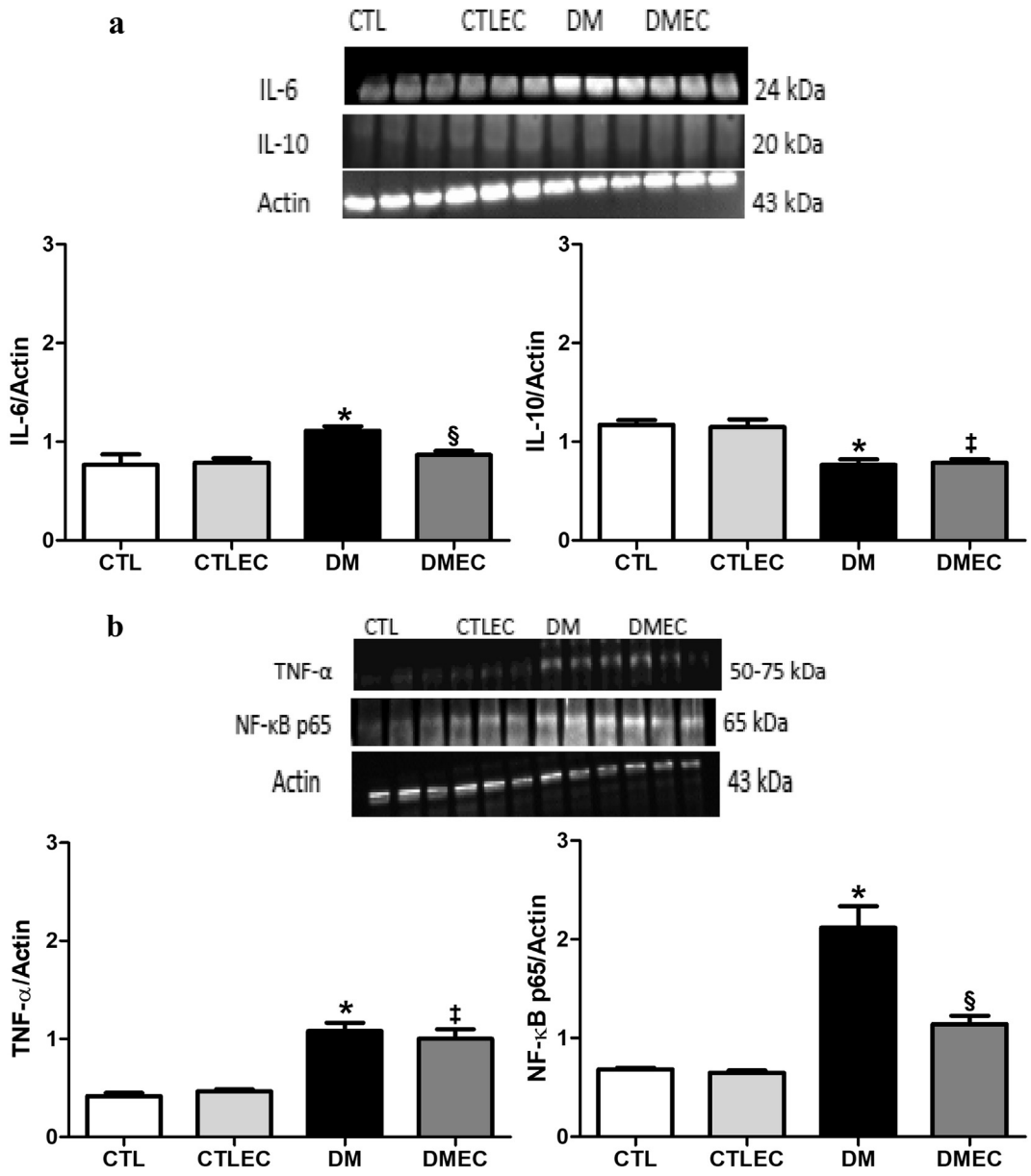

Fig. 4. IL-6 and IL-10 (**a**), TNF-α and NF-κB p65 (**b**) in the renal tissue after EC. IL: interleukin; TNF-α: tumor necrosis factor alpha; nuclear transcription factor kappa B; EC: extract of cupuaçu. Control (CTL); control plus EC (CTLEC); diabetic (DM); diabetic plus EC (DMEC); n = 4 per group. Values were expressed on average and SEM. ANOVA with Newman–Keuls or Kruskal Wallis with Dunn's; $P < 0.05$: *vs. CTL; ‡vs. CTLEC; §vs. DM.

strategies to interfere in the phases of NO oxidizing path to reduce the renal failure progression [24]. It is known that NO bioavailability is reduced in the chronic phase of the diabetes, probably due to increased oxidative/nitrosative stress induced by hyperglycemia [25].

In our study, superoxide anion was increased in the kidney of DM vs. CTL, with no statistic difference between DMEC vs. CTLEC and DM had increased levels of NO, showing a significant elevation in nitrosative stress showed by increased 3-nitrotyrosine and eNOS, which were reduced in DMEC. Factors such as high glycemia and oxidative/nitrosative stress can prevent the physiological signaling of NO, making it more susceptible to the environment in which it is inserted, contributing to deleterious actions in the organism [26]. These results agree with those mentioned by Ishii et al., showing that NOS is elevated in renal cortex of diabetic animals and as a consequence, more superoxide are produced [27]. Our data confirm the antioxidant action of the phenolic compounds contained in the cupuaçu and agree with the findings of other study, in which cupuaçu promoted antioxidant action in diabetic rats [12].

In addition, studies have demonstrated that the uncoupling of eNOS, caused by the oxidation of the eNOS co-factor through peroxynitrite, can be a secondary pathway by which hyperglycemia may inactivate NO and consequently, leading to endothelial dysfunction [28,29]. A randomized, double-blind study showed that epicatechins of cocoa and tea may contribute to the cardioprotective effects through improvement in endothelial function [30]. In our study, there was increase of eNOS in DM vs. CTL, revealing reduction of this parameter in DMEC, suggesting that reduction in nitrosative stress could reestablish the endothelial function or prevent its dysfunction in diabetic kidney [2,31].

High glucose levels also contribute to activate NF-κB, which stimulates the increase of NO production, favoring β-cell damage

[32]. NF-κB-dependent pathways play key role in the renal damage associated with cell proliferation, apoptosis, fibrosis, and inflammatory response [33]. Our results demonstrated diabetic animals had high production of NO and ROS, probably via upregulation of eNOS, 3-NT and NF-κB p65. Our data agree with another study that showed inhibition of NF-κB activation and inflammatory markers using polyphenols in cultured LPS-induced monocytes from healthy individuals [34]. Our findings showed a significant increase of NF-κB p65 in DM, characterizing the inflammatory response, and this was significantly reduced after EC treatment.

It is known that diabetes is a chronic inflammatory disease and hyperglycemic condition is one of the causes of elevated plasma concentrations of pro-inflammatory cytokines (TNF-α and IL-6) [35]. Moreover, there are still other oxidative/nitrosative stresses, which contribute to secretion of these cytokines, influencing the expression of several genes in vascular cells, and signaling molecules as NF-κB, favoring vascular damage [36,37].

Experiments with Caco-2 cells showed that cocoa polyphenols may downregulate inflammatory markers TNF-α-induced by inhibiting JNK phosphorylation and translocation of NF-κB and; besides, polyphenols could suppress inflammation-associated colon carcinogenesis [38]. Another study suggests that antioxidant compounds supplementation could be important to prevent or treat chronic inflammatory diseases through inhibition of the transcription factor NF-κB, and reduction of cytokines, pro-inflammatory chemokines and inflammatory mediators in healthy adults [34]. In our study, it was verified that in DM there was a significant increase of IL-6 and NF-κB p65 vs. CTL group; however, DMEC animals showed lower values of these markers, suggesting that the reduction in these inflammatory parameters could have occurred due to significant decrease in nitrosative stress caused by the intake of EC.

This study showed that EC treatment significantly reduced the nitrosative stress in MiMC incubated under high glucose by NO and ROS reduction, downregulating the inflammation by inhibition of NF-κB in kidneys of STZ-induced diabetic rats. EC also improved the renal function and some metabolic parameters in these animals. These findings suggest the importance of daily intake of the bioactive compounds rich in proanthocyanidins, such as cupuaçu, which could be an adjuvant non-pharmacological tool to reduce the diabetic complications triggered by nitrosative stress and inflammation in the kidneys.

In addition, the limitations of the present study are small study groups and short observational period, therefore, the results need to be verified in further observations, in the larger study groups.

Statement of authorship

GRP: delineate the study, conducted the experiments and statistical analysis and, wrote the manuscript. DYL and AMR: contributed to the planning of study, analysis of laboratory data and interpretation of the results. SP: helped in data collection and in the experimental protocol. MGM and MMR: helped in the experimental protocol and contributed to the manuscript. Elisa Mieko Suemitsu Higa supervised the protocol and reviewed this paper.

Conflict of interest

All the authors declared that they have no conflicts of interests.

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