Biomedicine & Pharmacotherapy 105 (2018) 95–102

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Naringin attenuates liver damage in streptozotocin-induced diabetic rats

Valeria Rodríguez[a], Luis Plavnik[b], Nori Tolosa de Talamoni[a,*]

[a] Laboratorio "Dr. Cañas", Cátedra de Bioquímica y Biología Molecular, Facultad de Ciencias Médicas, INICSA (CONICET-Universidad Nacional de Córdoba), Argentina
[b] Cátedra de Histología, Facultad de Odontología, Universidad Nacional de Córdoba, Córdoba, Argentina

ARTICLE INFO

Keywords:
Type 1 diabetes
Naringin
Liver
Oxidative stress
Inflammation
Nitrosative stress

ABSTRACT

The aim of this study was to evaluate whether NAR has a hepatoprotective role in a model of STZ-induced diabetes and to elucidate the underlying mechanisms triggered by the flavonoid. Male Wistar rats were divided into three groups: 1) controls, 2) STZ rats 3) STZ rats treated daily with NAR (40 mg/kg b.w.) for 30 days. NAR prevented increases in serum aminotransferases and alkaline phosphatase activities in STZ rats. The flavonoid blocked serum lipid alterations, but not the biometric parameters in STZ rats. Microscopic examination in liver from STZ rats revealed morphological changes indicative of increased adipogenesis and cell death and inflammation, which were all mitigated by the flavonoid. NAR inhibited the NFκB/IL-6/Cox-2 overexpressions triggered by oxidative stress in STZ rats. The iNOS/NO·/nitrosylated protein pathway was also blocked by NAR. The increment in the protein expression of Fas/FasL/caspase-3 and in the Bax/Bcl-2 ratio showed that both pathways of apoptosis were increased by the diabetes, effects that were abrogated by NAR treatment. In conclusion, NAR protects against the liver damage caused by STZ-induced diabetes and it could be a novel therapeutic strategy to prevent the non alcoholic fatty liver disease associated with the type 1Diabetes mellitus.

1. Introduction

Diabetes is a lifelong chronic disease, which is one of the most severe endocrine metabolic disorders worldwide [1]. Type 1 diabetes mellitus (T1DM) constitutes only around 10% of the diabetes cases, but occurs early in life [2] and becomes more prevalent each year [3]. Although the liver is a central organ in metabolism and has been recognized to be injured in this disease [4], the hepatic damage and dysfunction in T1DM have not been well addressed [5]. Nevertheless, it has been reported that patients show hepatomegaly, abdominal pain, and elevated serum levels of aspartate transaminase and alanine transaminase activities [6]. The elevations of these serum aminotransferases are indicative of the leakage of hepatic enzymes into circulation and are used as biomarkers in clinical practice to screen for liver disease [7]. Liver dysfunction is not commonly observed in infants and children with T1DM [8], but in a clinical audit in adults with T1DM, elevated liver enzymes were associated with a less favorable cardiovascular risk profile and poorer glycemic control [9].

The onset of diabetes in the liver is associated with the development of biochemical and functional abnormalities, including oxidative stress and apoptosis [10,11]. In addition, inflammation and necrosis or fibrosis of non-alcoholic fatty liver disease has been reported to follow diabetes [12]. Madar et al. [13] have also found that STZ-induced diabetes increased iNOS activity in the rat liver and in the isolated hepatocytes. Taurino et al. [14] have found that the diabetic liver presents abnormalities in the 14-3-3 proteins, which constitute a family of conserved molecular chaperones with roles in the regulation of metabolism, signal transduction, cell cycle control, protein trafficking and apoptosis.

Nowadays, medicinal plants have received attention to manage diabetes because they offer some hope and have less side-effects than the conventional medications [15]. A recent study has shown that higher habitual flavonoid intake from fruit and vegetable during adolescence is relevant for the prevention of risk factors of type 2 diabetes in early adulthood [16]. Naringin (NAR) is a flavonoid enriched in grapefruit, orange and tomatoes with antihyperlipidemic, antiapoptotic and antioxidant properties. It has been reported to be a useful nutraceutical in the management of diabetes and its complications [17]. We have recently showed that NAR avoids alterations in the physical properties and microstructure of bone from diabetic animals, probably by stimulation of osteoblastogenesis, inhibition of osteoclastogenesis and adipogenesis, at least in part via blocking the oxidative stress

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CAT, catalase; DAB, 3,3′-diaminobenzidine; GSH, glutathione; H&E, hematoxylin-eosin; HSI, hepatosomatic index; NAR, naringin; NBT, nitro blue tetrazolium; NO·, nitric oxide; $\cdot O_2^-$, superoxide anion; RIA, radioimmunoassay; RIPA, radioimmunoprecipitation assay buffer; SOD, superoxide dismutase; STZ, streptozotocin; T1DM, type 1 diabetes mellitus; TG, triglycerides

* Corresponding author at: Cátedra de Bioquímica y Biología Molecular, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Pabellón Argentina, 2do. Piso, Ciudad Universitaria, 5000, Córdoba, Argentina.
E-mail address: ntolosa@biomed.fcm.unc.ed (N. Tolosa de Talamoni).

https://doi.org/10.1016/j.biopha.2018.05.120
Received 10 April 2018; Received in revised form 23 May 2018; Accepted 23 May 2018

[18].The purpose of the present work was to evaluate whether NAR has a hepatoprotective role in a model of STZ-induced diabetes in rats and to elucidate the underlying mechanisms triggered by the flavonoid.

2. Material and methods

2.1. Chemicals

All chemicals were purchased from Sigma Aldrich Co (St Louis, MO, USA), unless otherwise stated.

2.2. Animals and experimental design

Male Wistar rats (150–200 g) were maintained at 20–25 °C in a 12 h light/12 h dark cycle, with a commercial normal rodent diet (GEPSA mouse-rat, Pilar, Buenos Aires, Argentina) and water freely available. They were divided into three groups: 1) control rats, 2) STZ-induced diabetic rats, 3) STZ-induced diabetic rats + NAR: three days after STZ injection, this group of rats received daily NAR (40 mg /kg b.w., dissolved by sequential addition of 5% DMSO and 95% sterile normal saline) therapy via subcutaneous injection. Rats from the second and third groups received a single intraperitoneal injection of STZ (60 mg/kg b.w. dissolved in 0.1 mol/L citrate, pH 4.5 solution), whereas the control rats were injected with vehicle alone. After 3 days of STZ injection, the blood glucose levels were measured by using a glucometer (AccuCheck; Roche Diagnostics, Mannheim, Germany). Rats were considered diabetic when their blood glucose values exceeded 250 mg/dL and glucose was detected in urine (Multistix, Siemens Medical Solutions Diagnostics, Malvern, USA). After 30 days of treatment and an overnight fast, they were weighed and killed by cervical dislocation for analysis of liver tissues. The hepatosomatic index (HSI) was calculated as liver mass × 100 divided by the rat final body weight. In some experiments other NAR doses were used (see Results).

The studies were conducted according to the Guide for Care and Use of Laboratory Animals. The protocol was approved by the CICUAL (Res. 09/16, Commission for Care and Use of Laboratory Animals, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Córdoba, Argentina). All efforts were made to minimize the number of animals used and their suffering.

2.3. Serum biochemical analysis

Blood samples from rats were used for biochemical measurements. Serum glucose (Glicemia enzimática AA), triglycerides (TG Color, GPO/PAP AA), cholesterol (Colestat enzimático AA), ALT (alanine aminotransferase, ALT-UV AA), AST (aspartate aminotransferase, AST-UV AA), ALP (alkaline phosphatase, ALP 405AA), were determined using kits from Wiener Laboratorios S.A.I.C. (Rosario, Argentina) following the manufacturer´s protocol. Serum insulin was measured by radioimmunoassay (RIA) using an anti-rat insulin antibody (Sigma, St. Louis, Missouri, USA) and HbA_{1c} was determined by Glycohemoglobin Reagent (Teco Diagnostics, Anaheim, CA, USA), according to manufacturer's operating protocol.

2.4. Histological preparation

For histological examination, the hepatic tissues of each group were fixed in 10% buffered formalin, and then cut in segments to perform dehydration in graded alcohol series, clearance with xylene, embedding in paraffin, and sectioning at 4 µm slices. Liver sections were stained with hematoxylin-eosin (H&E). Images were obtained with a Leica DC 180 Camera (software Leica IM50 Image Manager; Leica, Cambridge, UK) using a 40× objective lens magnification. The H&E stained were used for histomorphometric studies and counting of the number of apoptotic nuclei (Nº apoptotic nuclei/mm^2) using Image Pro Plus 4.5 software (Media Cybernetics, Inc. Rockville, MD 20850, USA). Condensed chromatin near the periphery of the nuclei was used as a criterion to identify apoptosis. The average diameter of each cell was calculated as the mean of the largest and smallest diameters. Two independent operators observed the sections and did the counting under the microscope.

2.5. Glutathione (GSH) content

Total GSH content was determined in liver homogenates using the glutathione disulfide reductase-5,5′-dithiobis (2-nitrobenzoate) recycling procedure, as described elsewhere [19]. The data are expressed in nmol/mg of protein.

2.6. Superoxide anion ($\cdot O2^-$) measurement

The liver was washed twice with Hanks buffer (137 mmol/L NaCl, 5.4 mmol/L KCl, 0.25 mmol/L Na_2HPO_4, 0.4 mmol/L KH_2PO_4, 1.3 mmol/L $CaCl_2$, 1 mmol/L $MgSO_4$, 4.2 mmol/L $NARHCO_3$, 6.24 mmol/L glucose, pH 7.4) and incubated with nitro blue tetrazolium (NBT) (1 mg/mL) at 37 °C for 1 h. The formazan precipitates formed were dissolved in dimethylsulfoxide and quantified by spectrophotometry at 560 nm. OD values are direct indicators of $\cdot O_2^-$ concentration in the samples [20].

2.7. Catalase and superoxide dismutase activities

All enzymes activities were assayed in supernatants of liver homogenates. Catalase (CAT), EC 1.11.1.6, and superoxide dismutase (Mg^{2+}-SOD), EC 1.15.1.1, activities were performed in diluted aliquots of supernatants from liver homogenates (1:5). CAT activity was assayed in 50 mmol/L potassium phosphate buffer pH 7.4 and 0.3 mol/L H_2O_2 [21]. Mg^{2+}-SOD activity was determined in 1 µmol/L EDTA, 50 mmol/L potassium phosphate buffer, pH 7.8, 13 mmol/L methionine, 75 µmol/L NBT and 40 µmol/L riboflavin [22]. The results of enzyme activities are expressed in U/mg of protein.

2.8. Nitric oxide levels

The levels of nitric oxide (NO·) were detected as total nitrate/nitrite using the Griess reagent [23] with the modification of replacing zinc sulfate by ethanol for protein precipitation in the liver homogenate supernatant [24]. A standard curve of sodium nitrate was used (1–10 µmol/L). The absorbance was read at 540 nm and the data were expressed as µmol NO·/mg of protein.

2.9. Preparation of nuclear fraction

Nuclear fraction was isolated from the liver of each group of rats by differential centrifugation. Liver tissue was resuspended in 1 mL of ice-cold buffer A (230 mmol/L mannitol, 70 mmol/L sucrose, 1 mmol/L EDTA, 5 mmol/L TrisCl) and homogenized. The homogenate was centrifuged at 1200 × *g* for 20 min at 4 °C. The supernatant was centrifuged at 2200 × *g* for 20 min at 4 °C, and the pellet was lysed in 1 mL of ice-cold buffer B (230 mmol/L mannitol, 70 mmol/L sucrose, 5 mmol/L TrisCl) and was centrifuged at 3000 × *g* for 20 min at 4 °C. The nuclei then were extracted with 500 µL of ice-cold buffer B and were stored at −80 °C.

2.10. Western blot analysis

Liver tissues were homogenized in RIPA (radioimmunoprecipitation assay buffer) lysis buffer (1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate in PBS, containing 1 mmol/L PMSF and 1 mmol/L NaF), and then centrifuged. Afterwards, proteins (100 µg) were denatured for 5 min. at 95 °C and fractionated in 12% (w/v) SDS–polyacrylamide minigels for caspase-3, Bax, Bcl-2, Fas, FasL, IL-6 and in 8% (w/v)

SDS–polyacrylamide minigels for the other proteins [25]. Gels containing the separated proteins were immersed in a transfer buffer (25 mmol/L Tris–HCl, and 192 mmol/L glycine, 0.05% w/v SDS and 20% v/v methanol) [26]. Nitrocellulose membranes (0.45 μm) were blocked for 1.5 h with 2% w/v nonfat dry milk in 0.5 mol/L Tris-buffered saline solution and incubated overnight at 4 °C with the specific primary antibody at 1:200 (Bax and caspase-3) and at 1:1000 dilution for the other proteins. The antibodies were: rabbit anti-active caspase-3 monoclonal antibody (BD Pharmigen Biosciences, San Jose, CA, USA), rabbit anti-Bax polyclonal antibody (P-19, Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-Bcl-2 monoclonal antibody (BD Pharmigen Biosciences, San Jose, CA, USA), mouse anti-CD95 monoclonal antibody (BD Pharmigen Biosciences, San Jose, CA, USA), mouse anti-FasL monoclonal antibody (N-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-NOS2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-nitrotyrosine monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-NFκB p50 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-IL-6 monoclonal antibody (C12-1-hIL-6, Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-Cox-2 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After three washings, appropriate secondary biotinylated antibodies were incubated at room temperature for 1 h. Then, the blots were washed three times and streptavidin - biotin conjugate (Histostain-SP Broad Spectrum, Invitrogen, Carlsbad, CA, USA) was added. Detection was performed using 3,3′-diaminobenzidine (DAB) as a chromogen. Monoclonal antibody anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), was used to detect β-actin as a marker to normalize the relative expression of the other proteins. The membranes were stripped in stripping buffer (0.5 M acetic acid, 0.5 M NaCl pH = 2.6) for 10 min at room temperature before to probe for loading control. The band intensities were quantified using an Image Capturer EC3 Imaging System, LaunchVisionworksls software in order to obtain the relative expression of proteins.

2.11. Statistical analysis

The data were obtained from at least 3 different experiments and were expressed as means ± S.E. Results were evaluated by one-way analysis of variance (ANOVA) and the Bonferroni's test as a post hoc test. Differences were considered statistically significant at $p < 0.05$. All the analyses were carried out by using SPSS software (version 22.0) for Windows 8.1 (SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. NAR prevention of serum ALT, AST and ALP increase in STZ rats was dose dependent

As can be seen in Table 1, the increment in the activities of serum ALT, AST and ALP indicates that the hepatic dysfunction occurred after 30 days of STZ injection. Although those increases were reduced by the daily injection of 10 mg NAR/kg b.w., the values did not return to the normal ones. Doses of 40 mg NAR/kg b.w. or above were capable to avoid the augmentation in the enzyme activities and the values were normalized, except those of ALP. Based on these data, the following studies were accomplished using a dose of 40 mg NAR/kg b.w.

3.2. NAR blocked serum lipid alterations but not the biometric parameters and other metabolic variables in STZ rats

As expected, the diabetic rats exhibited a decrease in the body weight and a noticeable increase in HSI after 30 days of STZ injection. NAR was not able to avoid these changes. Serum insulin was diminished to one fourth of the normal values and, consequently, serum glucose increased four times and HbA_{1c} was increased about 50%, and NAR could not prevent these alterations in the diabetic rats. However, an increase in the serum total cholesterol of STZ rats was completely abrogated by NAR treatment. Although the flavonoid could avoid the enormous increment in the serum TG of STZ rats, it was not able to normalize the values (Table 2).

Table 1
Serum aminotransferases and alkaline phosphatase from control, STZ-induced diabetic rats and STZ rats treated with NAR.

Groups	ALT (U/L)	AST (U/L)	ALP (U/L)
Control	44.34 ± 1.39	108.55 ± 3.85	420.19 ± 30.20
STZ	152.30 ± 18.30*	208.91 ± 8.32*	2981.70 ± 40.10*
STZ + NAR10	93.35 ± 3.48#	215.16 ± 7.65	1890.30 ± 357.70+
STZ + NAR40	46.32 ± 0.68	112.85 ± 3.25&	1946.02 ± 281.11+
STZ + NAR80	43.74 ± 1.86	111.04 ± 1.51&	1845.10 ± 265.40+

Values represent means ± S.E. from 5 rats for each experimental condition.
* $p < 0.001$ vs control.
# $p < 0.05$ vs STZ, STZ + NAR40 and STZ + NAR80.
& $p < 0.001$ vs STZ and STZ + NAR10.
+ $p < 0.001$ vs STZ. STZ: streptozotocin induced diabetic rats; NAR: naringin (10, 40, 80 mg/kg b.w.); ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase.

Table 2
Biometric data and serum biochemical parameters.

	Control	STZ	STZ + NAR
Body weight (g)	263.33 ± 5.40	180.26 ± 4.17*	188.32 ± 5.15
HSI (g/100 g)	3.30 ± 0.09	4.85 ± 0.15*	5.52 ± 0.11
Serum Glucose (mg/dL)	120.75 ± 24.14	536.00 ± 20.75*	517.00 ± 11.19
Serum Insulin (ng/mL)	1.59 ± 0.02	0.40 ± 0.03*	0.44 ± 0.03
HbA_{1C} (%)	8.53 ± 0.45	12.01 ± 0.93*	11.93 ± 0.85
TG (mg/dL)	93.37 ± 7.80	536.97 ± 27.28*	274.17 ± 34.28+
Total cholesterol (mg/dL)	79.53 ± 3.07	103.14 ± 1.12*	80.90 ± 1.18+

Values are expressed as means ± S.E. from 8 rats for each experimental condition.
* $p < 0.001$ vs control.
+ $p < 0.001$ vs STZ. STZ: streptozotocin-induced diabetic rats; NAR: naringin; HSI: hepatosomic index; TG: triglycerides.

Table 3
Morphometric parameters in liver from control, STZ-induced diabetic rats and STZ rats treated with NAR.

	Control	STZ	STZ + NAR
Nº hepatocytes/ test area	7.95 ± 0.08	6.59 ± 0.10*	7.65 ± 0.16+
Hepatocyte cell area (μm^2)	245.30 ± 5.20	288.76 ± 4.33*	242.92 ± 4.19+
Hepatocyte cell diameter (μm)	31.33 ± 0.82	41.42 ± 1.35*	30.95 ± 0.11+
Hepatocyte nuclear area (μm^2)	52.23 ± 0.82	65.11 ± 0.82*	53.98 ± 1.10+
Hepatocyte nuclear diameter (μm)	6.85 ± 0.82	8.21 ± 0.43*	6.73 ± 0.91+

Values are expressed as means ± S.E. from 8 rats for each experimental condition.
* $p < 0.001$ vs control.
+ $p < 0.001$ vs STZ. STZ: streptozotocin induced diabetic rats; NAR: naringin (40 mg/kg b.w.).

Table 4
Total GSH content, superoxide anion, SOD and CAT activities in liver from control, STZ- induced diabetic rats and STZ rats treated with NAR.

Groups	GSH (nmol/mg protein)	$\cdot O_2^-$ (NBT reduction, OD)	SOD (SOD U/mg protein)	CAT (CAT U/mg protein)
Control	15.34 ± 0.62	0.79 ± 0.10	59.08 ± 7.98	2.15 ± 0.19
STZ	7.88 ± 0.55*	1.28 ± 0.06*	101.20 ± 3.94*	38.84 ± 1.62*
STZ + NAR	11.61 ± 0.46+	0.93 ± 0.04+	71.06 ± 3.87+	17.62 ± 2.25+

Values represent means ± S.E. from 5 rats for each experimental condition.
* $p < 0.001$ vs control.
+ $p < 0.001$ vs STZ. STZ: streptozotocin- induced diabetic rat; NAR: naringin (40 mg/kg b.w.); GSH: glutathione; $\cdot O_2^-$: superoxide anion; NBT: nitro blue tetrazolium; OD: optical density; SOD: superoxide dismutase; CAT: catalase.

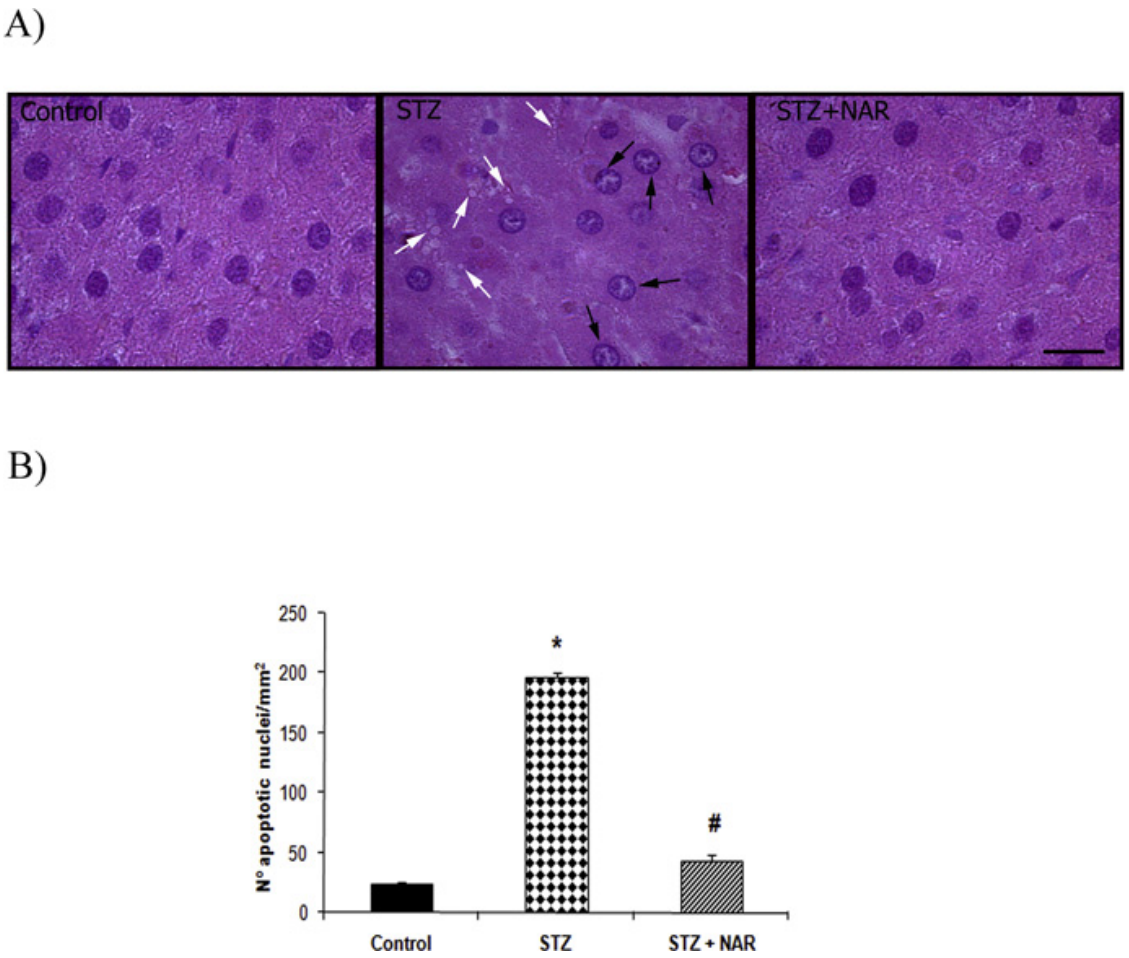

Fig. 1. A) Liver sections from control, STZ and STZ + NAR rats stained with H&E. The black arrows indicate the apoptotic cells and the white arrows indicate the lipid droplets as shown in the inset. Scale bars = 50 μm; **B)** Number of apoptotic nuclei/mm^2 in the liver (n = 8). Values are expressed as means ± S.E. *$p < 0.001$ *vs* control; #$p < 0.01$ vs STZ. STZ: streptozotocin induced diabetic rats; NAR: naringin (40 mg/kg b.w.).

3.3. Changes in hepatic morphometric parameters in STZ-induced diabetic rats were avoided by NAR

The number of hepatocytes/test area was decreased in STZ-induced diabetic rats, which was prevented by NAR injection. The cell and nuclear diameters as well as the cell and nuclear areas of hepatocytes from diabetic rats were increased, effects that were also avoided by the flavonoid (Table 3).

3.4. NAR mostly inhibited variables involved in the hepatic redox state

Table 4 shows that STZ-induced diabetic rats presented hepatic oxidative stress as indicated by a lower GSH content, higher $\cdot O_2^-$ as well as higher SOD and CAT activities compared to those from the control rats. NAR administration partially avoided the changes in the GSH content and in the CAT activity, and completely blocked the alterations in the $\cdot O_2^-$ content and in the SOD activity.

3.5. Histological and biochemical changes in hepatic tissue associated with apoptosis in diabetic rats were highly avoided by NAR injection

As depicted in Fig. 1A, histological sections of liver from STZ rats showed extensive regions with loss of hepatic architecture, dilatation of hepatic sinusoid capillaries close to the central vein, apoptotic cells and lipid droplets inside of many cells. These alterations were not seen in the liver from STZ rats treated with NAR. The number of apoptotic nuclei/mm^2 in the liver from STZ rats was strikingly increased in comparison with that of the control rats, and NAR treatment avoided

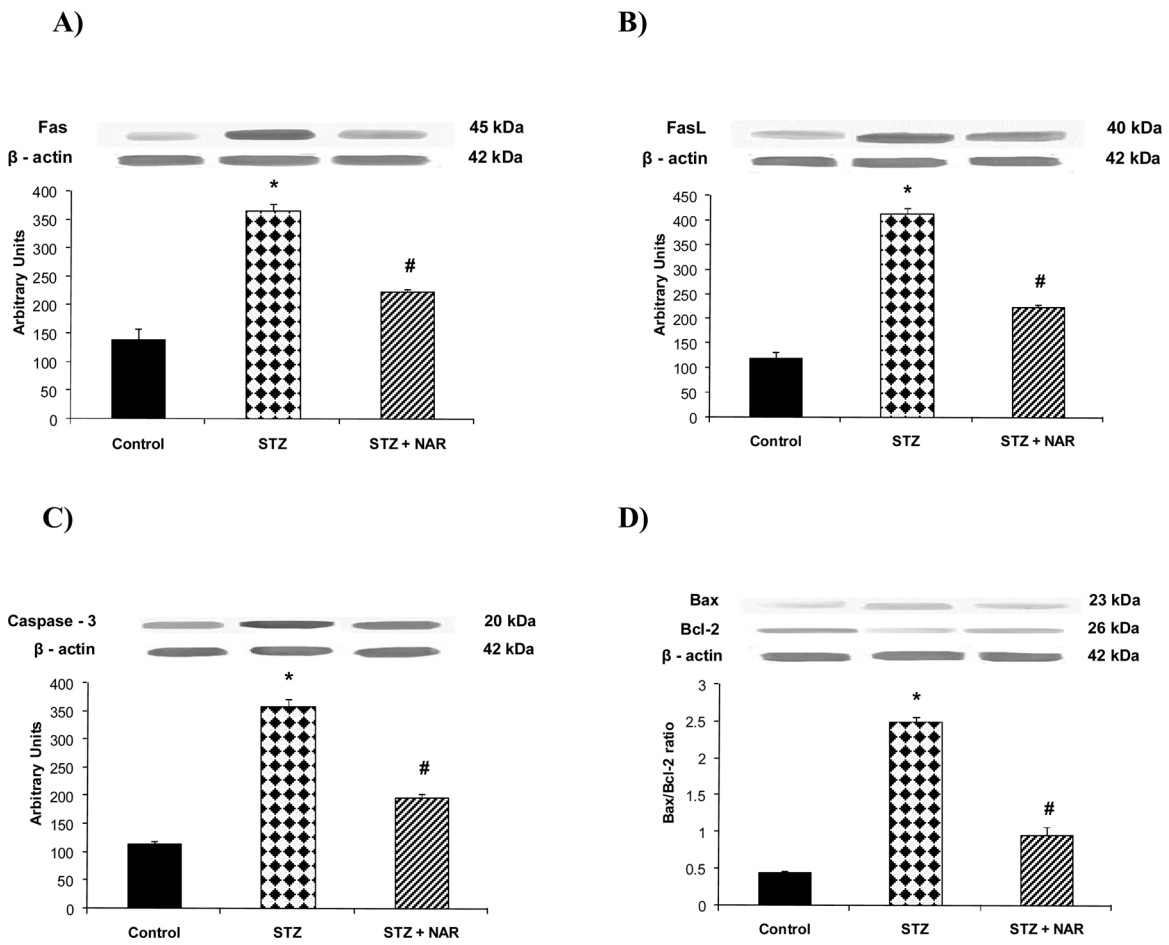

Fig. 2. The protein expression of Fas (**A**), FasL (**B**), caspase-3 (**C**), and Bax/Bcl2 ratio (**D**) was analyzed by Western blot in liver homogenate for each experimental condition: control, STZ and STZ + NAR (40 mg NAR/kg b.w.). (n = 6 per group). Values are expressed as means ± S.E. *p < 0.001 vs control; #p < 0.01 vs STZ. STZ: streptozotocin induced diabetic rats; NAR: naringin.

those changes, but it was not able to normalize the parameters (Fig. 1B). The biochemical variables involved in both the extrinsic and intrinsic pathways of apoptosis were also altered in the liver from the STZ- induced diabetic rats, as shown by the increment in the protein expression of Fas/FasL/caspase-3 as well as that from Bax/Bcl-2 ratio. Although NAR could partially avoid this response, the values remained higher than the corresponding to those from the control rats (Fig. 2).

3.6. NAR was successful to inhibit the alterations in the nitrergic system caused by STZ

NO• content and the protein expression of iNOS were enhanced in liver from STZ induced diabetic rats, which was completely abolished by NAR treatment. The nitrotyrosine content of protein bands with 75 kDa and 30 kDa was increased by STZ, which was also totally avoided by NAR injection (Fig. 3).

3.7. NAR blocked the increment in the protein expression of molecules involved in the hepatic inflammation caused by STZ

After 30 days of injection, STZ produced a significant increase in the protein expression of NFκB in the nuclei, and in those of IL-6 and Cox-2 of homogenates from rat liver, which were inhibited by NAR treatment (Fig. 4).

4. Discussion

The present data suggest that NAR has a protective role in the liver injury caused by STZ-induced diabetes in rats, as demonstrated by blocking morphological and biochemical changes caused by this disease. The microscopic examination of liver tissue from STZ rats revealed loss of hepatic architecture, dilatation of hepatic sinusoid capillaries close to the central vein, apoptotic hepatocytes and hepatocytes with lipid droplets in their cytoplasm indicating increased adipogenesis and cell death as well as signs of inflammation, which were all mitigated by NAR. In addition, STZ caused an increase in the size of the hepatocytes and in their nuclei, but not a decrease in the nucleus-to-plasma ratio as found by others [27]. NAR was also able to avoid these size changes in hepatocytes. Furthermore, the systemic increases of ALT, ASP and ALP in diabetic animals are other indications of liver disease associated with T1DM, which has been previously shown in children and in animals [1,28–30]. The increased activity of AST and ALT in liver tissue suggests defective utilization of glucose. The insulin deficiency leads to breakdown of protein and enhancing amino acid catabolism to provide substrates for gluconeogenesis [31]. NAR has the ability to ameliorate the activity of systemic transaminases in a dose dependent manner that would lead to an improvement in the hepatic dysfunction. However, the circulating ALP activity decreases after NAR treatment, but it remains very high independently of the dose employed. It is quite possible that longer times of exposition or higher NAR concentration could normalize the ALP activity. ALT half-life in circulation is about 47 h, for total AST is 17 h and for mitochondrial AST is 87 h. In contrast, ALP half-life in circulation is about 7 days [32]. These differences might also contribute to explaining why ALP levels decrease slowly after NAR treatment.

STZ-induced diabetic rats exhibited a significant loss of body mass

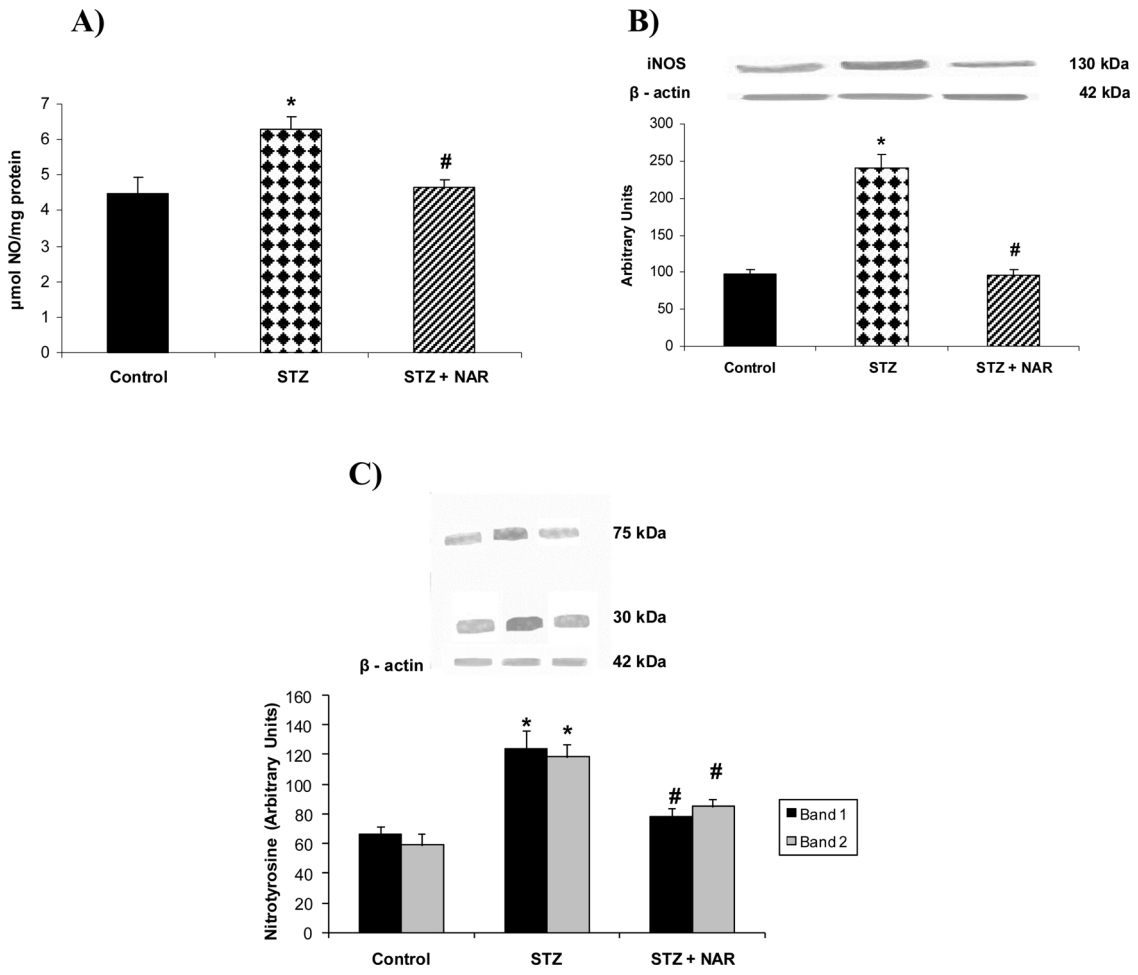

Fig. 3. Effect of vehicle, STZ or STZ + NAR (40 mg NAR/kg b.w.) on: **A)** NO· level measured by Griess assay in the liver from 5 rats for each experimental condition. Values are expressed as means ± S.E. *p < 0.01 vs control and STZ + NAR; **B)** iNOS expression, and **C)** nitrotyrosine expression was analyzed by Western blot in liver homogenate. (n = 6 per group). Values are expressed as means ± S.E. *p < 0.01 vs control. #p < 0.01 vs STZ. STZ: streptozotocin induced diabetic rats; NAR: naringin.

compared to controls, which is a known phenomenon in children and young adults with T1DM [33]. The weight loss did not disappear under NAR treatment in STZ rats. In contrast, Murunga et al. [15] have found that NAR increased weight gain compared to diabetic rats. Differences in animal strain and/or timing of NAR treatment might explain these differences.

As previously shown [18], NAR does not normalize hyperglycemia, hypoinsulinemia and the high levels of HbA_{1c}, which suggests that the NAR action on liver dysfunction is not related to systemic variables associated with the glucose metabolism. However, NAR trends to normalize the dyslipidemia in rats with STZ induced diabetes. Serum cholesterol goes down to normal values and TG decreases without reaching the control ones. Similarly, Xulu and Oroma Owira [34] have found that NAR ameliorates atherogenic dyslipidemia, but not hyperglycemia in rats with type 1 diabetes. Murunga et al. [15] have also demonstrated that NAR is not hypoglycemic but mitigates ketoacidosis and lipid peroxidation in type 1 diabetes rat model. Contrarily, NAR has been shown to prevent the progression of hyperglycemia in type 2 diabetic db/db mice, at least in part, by increasing hepatic glycolysis and glycogen content and lowering hepatic gluconeogenesis [35].

It has been suggested that the oxidative stress contributes to the onset and progression of higher incidence of liver disease in diabetic patients [36]. Oxidative stress is a result of an imbalance between ROS production and the antioxidant defenses. Our data indicate that the main players in inducing oxidative stress in the liver from diabetic rats appear to be an enhancement in the $\cdot O_2^-$ content and a decrease in the GSH levels. Although SOD and CAT increase their activities in order to neutralize ROS, this is not enough for a total compensation and, therefore, the oxidative stress predominates in the diabetic condition. NAR is able to prevent all these variables altered by STZ, except the hepatic CAT activity that remains very high in the presence of the flavonoid.

The diabetic rats exhibited elevated levels of NFκB, Cox-2 and IL-6 in the liver, effects that were blocked by NAR treatment. It is known that the oxidative stress associated with diabetes activates NFκB [37]. Following activation, NFκB translocates from the cytoplasm to the nucleus, where it binds with DNA and promotes the transcription of pro-inflammatory cytokines [38]. NFkB is a transcriptional regulator of inducible expression of genes including IL-6 [39] and Cox-2 genes [40]. NAR probably inhibits NFκB activation by scavenging ROS that act as signaling molecules to activate the NFκB pathway, as was suggested for other antioxidants [41]. In addition, NFκB promotes iNOS gene expression and the subsequent formation of NO· increasing the nitrosylation of proteins. In agreement with this, the STZ induced diabetic rats showed enhancement of hepatic NO· content and protein expression of iNOS as well as increase in the nitrotyrosine content of protein bands with 75 kDa and 30 kDa. Again, these alterations were completely abrogated by NAR treatment through mechanisms that need to be explored. The activation of NFκB-iNOS-NO· signaling pathway has been shown to be involved in the progressive loss and apoptosis of pancreatic β-cells in T1DM [42]. Similarly, our data indicate that the alteration in the nitrergic system in liver from diabetic animals is

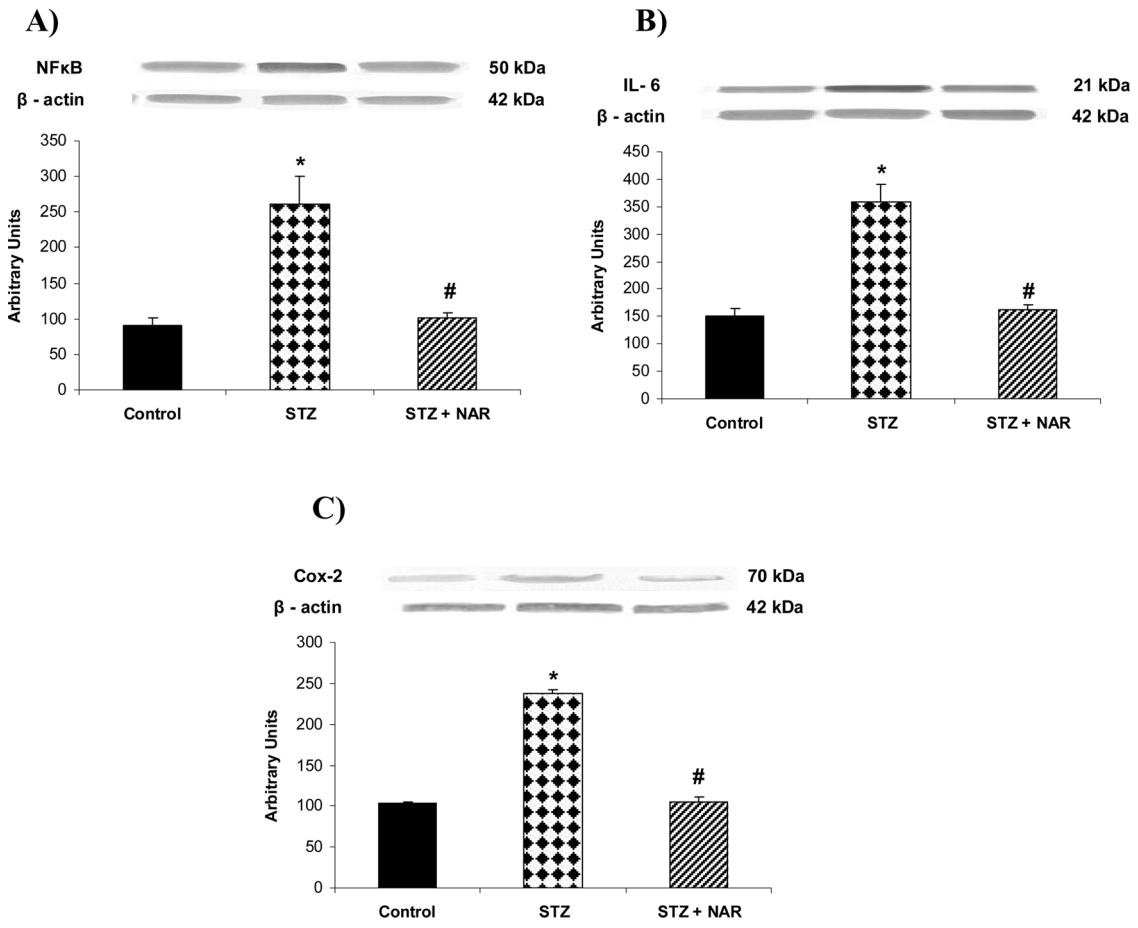

Fig. 4. The protein expression of NFκB p50 in nuclear extract (**A**), IL-6 in liver homogenate (**B**) and Cox-2 in liver homogenate (**C**) was analyzed by Western blot for each experimental condition: control, STZ and STZ + NAR (40 mg NAR/kg b.w.). (n = 6 per group). Values are expressed as means ± S.E. *$p < 0.01$ vs control. #$p < 0.01$ vs STZ. STZ: streptozotocin induced diabetic rats; NAR: naringin.

associated with an increase in the number of apoptotic hepatocytes, which is reinforced by increases in biochemical variables involved in both the extrinsic and intrinsic pathways of apoptosis, as demonstrated by the increment in the protein expression of Fas/FasL/caspase-3 as well as that from Bax/Bcl2 ratio. The antiapoptotic capability of NAR is not enough to restore the cells to the normality, which suggests that the protector mechanisms of NAR against the liver injury in the diabetic condition are mainly given by its antioxidant, antinitrergic and anti-inflammatory properties.

In conclusion, NAR protects against the liver damage caused by STZ induced diabetes through an inhibition of the NFκB/IL-6/Cox-2 over-expressions triggered by oxidative stress. In addition, the iNOS/NO ·/nitrosylated protein pathway, probably induced by NFκB, is also blocked by NAR showing its anti-nitrergic properties. The concomitant oxidative/nitrosative stress and inflammation provoked by the diabetic condition leads to an exacerbation of apoptotic hepatocytes, which could be partially ameliorated by NAR. Therefore, NAR could be a novel therapeutic strategy to prevent the non alcoholic fatty liver disease associated with the T1DM.

Acknowledgements

This work was supported by Grants from CONICET (PIP 2013-15) and SECYT (UNC), Argentina. Prof. Dr. Nori Tolosa de Talamoni and Dr. Valeria Rodríguez are Members of Investigator Career from the Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET). All authors participated in the conception, design, and performance of the study as well as the interpretation of data and drafting the manuscript. None of the authors had a personal conflict of interest. The authors thank Felipe Avila, student of the School of Medicine (UNC), for helping with some experiments.

References

[1] T. Liang, Q. Zhang, W. Sun, Y. Xin, Z. Zhang, Y. Tan, S. Zhou, C. Zhang, L. Cai, X. Lu, M. Cheng, Zinc treatment prevents type 1 diabetes-induced hepatic oxidative damage, endoplasmic reticulum stress, and cell death, and even prevents possible steatohepatitis in the OVE26 mouse model: important role of metallothionein, Toxicol. Lett. 233 (2015) 114–124.

[2] S.A. Paschou, A. Petsiou, K. Chatzigianni, A. Tsatsoulis, G.K. Papadopoulos, Type 1 diabetes as an autoimmune disease: the evidence, Diabetologia 57 (2014) 1500–1501.

[3] A.K. Brinkman, Management of type 1 diabetes, Nurs. Clin. North Am. 52 (2017) 499–511.

[4] S.A. Harrison, Liver disease in patients with diabetes mellitus, J. Clin. Gastroenterol. 40 (2006) 68–76.

[5] W. Wang, C. Wang, X.Q. Ding, Y. Pan, T.T. Gu, M.X. Wang, Y.L. Liu, F.M. Wang, S.J. Wang, L.D. Kong, Quercetin and allopurinol reduce liver thioredoxin-interacting protein to alleviate inflammation and lipid accumulation in diabetic rats, Br. J. Pharmacol. 169 (2013) 1352–1371.

[6] M. Torbenson, Y.Y. Chen, E. Brunt, O.W. Cummings, M. Gottfried, S. Jakate, Y.C. Liu, M.M. Yeh, L. Ferrell, Glycogenic hepatopathy: an underrecognized hepatic complication of diabetes mellitus, Am. J. Surg. Pathol. 30 (2006) 508–513.

[7] M.A. Gómez-Sámano, D. Cuevas-Ramos, R. Mehta, H. Brau-Figueroa, C.E. Meza-Arana, A. Gulias-Herrero, Association of alanine aminotransferase levels (ALT) with the hepatic insulin resistance index (HIRI): a cross-sectional study, BMC Endocr. Disord. 4 (2012) 12–16.

[8] L. Carcione, F. Lombardo, M.F. Messina, M. Rosano, F. De Luca, Liver glycogenosis as early manifestation in type 1 diabetes mellitus, Diabetes Nutr. Metab. 16 (2003) 182–184.

[9] M. Stadler, E. Bollow, M. Fritsch, W. Kerner, I. Schuetz-Fuhrmann, D. Krakow, S. Merger, M. Riedl, P. Jehle, R.W. Holl, DPV initiative and the german BMBF competence network diabetes mellitus. Prevalence of elevated liver enzymes in

adults with type 1 diabetes: a multicentre analysis of the German/Austrian DPV database, Diabetes Obes. Metab. 19 (2017) 1171–1178.
[10] P. Palsamy, S. Sivakumar, S. Subramanian, Resveratrol attenuates hyperglycemia-mediated oxidative stress, proinflammatory cytokines and protects hepatocytes ultrastructure in streptozotocin-nicotinamide-induced experimental diabetic rats, Chem. Biol. Interact. 186 (2010) 200–210.
[11] P.I. Ingaramo, M.T. Ronco, D.E. Francés, J.A. Monti, G.B. Pisani, M.P. Ceballos, M. Galleano, M.C. Carrillo, C.E. Carnovale, Tumor necrosis factor alpha pathways develops liver apoptosis in type 1 diabetes mellitus, Mol. Immunol. 48 (2011) 1397–1407.
[12] R. Afrin, S. Arumugam, M.I. Wahed, V. Pitchaimani, V. Karuppagounder, R. Sreedhar, M. Harima, H. Suzuki, S. Miyashita, T. Nakamura, K. Suzuki, M. Nakamura, K. Ueno, K. Watanabe, Attenuation of endoplasmic reticulum stress-mediated liver damage by mulberry leaf diet in streptozotocin-induced diabetic rats, Am, J. Chin. Med. 44 (2016) 87–101.
[13] Z. Madar, S. Kalet-Litman, A.H. Stark, Inducible nitric oxide synthase activity and expression in liver and hepatocytes of diabetic rats, Pharmacology 73 (2005) 106–112.
[14] F. Taurino, E. Stanca, L. Vonghia, L. Siculella, A.M. Sardanelli, S. Papa, F. Zanotti, A. Gnoni, Short-term type-1 diabetes differentially modulates 14-3-3 proteins in rat brain and liver, Eur. J. Clin. Invest. 44 (2014) 350–358.
[15] A.N. Murunga, D.O. Miruka, C. Driver, F.S. Nkomo, S.Z. Cobongela, P.M. Owira, Grapefruit derived flavonoid naringin improves ketoacidosis and lipid peroxidation in type 1 diabetes rat model, PLoS One 11 (2016) e0153241.
[16] K.J. Penczynski, C. Herder, D. Krupp, J. Rienks, S. Egert, S.A. Wudy, M. Roden, T. Remer, A.E. Buyken, Flavonoid intake from fruit and vegetables during adolescence is prospectively associated with a favourable risk factor profile for type 2 diabetes in early adulthood, Eur. J. Nutr. (2018), http://dx.doi.org/10.1007/s00394-018-1631-3.
[17] S.O. Rotimi, I.B. Adelani, G.E. Bankole, O.A. Rotimi, Naringin enhances reverse cholesterol transport in high fat/low streptozocin induced diabetic rats, Biomed. Pharmacother. 101 (2018) 430–437.
[18] M. Rivoira, V. Rodríguez, G. Picotto, R. Battaglino, N. Tolosa de Talamoni, Naringin prevents bone loss in a rat model of type 1 diabetes mellitus, Arch. Biochem. Biophys. 637 (2018) 56–63.
[19] M.E. Anderson, Determination of glutathione and glutathione disulfide in biological samples, Methods Enzymol. 113 (1985) 548–555.
[20] L. Serrander, L. Cartier, K. Bedard, B. Banfi, B. Lardy, O. Plastre, A. Sienkiewicz, L. Fórró, W. Schlegel, K.H. Krause, NOX4 activity is determined by mRNA levels and reveals a unique pattern of ROS generation, Biochem. J. 406 (2007) 105–114.
[21] H. Aebi, S.R. Wyss, B. Scherz, F. Skvaril, Catalase, Eur. J. Biochem. 48 (1974) 137–145.
[22] C.O. Beauchamp, I. Fridovich, Isozymes of superoxide dismutase from wheat germ, Biochim. Biophys. Acta 317 (1973) 50–64.
[23] K.M. Miranda, M.G. Espey, D.A. Wink, A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite, Nitric Oxide 5 (2001) 62–71.
[24] H.H. Arab, S.A. Salama, A.H. Eid, H.A. Omar, S.A. Arafa, I.A. Maghrabi, Camel's milk ameliorates TNBS-induced colitis in rats via downregulation of inflammatory cytokines and oxidative stress, Food Chem. Toxicol. 69 (2014) 294–302.
[25] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[26] H. Towbin, T. Staehelin, J. Gordon, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, Proc. Natl. Acad. Sci. U. S. A. 76 (1979) 4350–4354.
[27] K. Welt, J. Weiss, R. Martin, D. Dettmer, T. Hermsdorf, K. Asayama, S. Meister, G. Fitzl, Ultrastructural, immunohistochemical and biochemical investigations of the rat liver exposed to experimental diabetes und acute hypoxia with and without application of Ginkgo extract, Exp. Toxicol. Pathol. 55 (2004) 331–345.
[28] I.M. Talaat, A. Nasr, A.A. Alsulaimani, H. Alghamdi, K.A. Alswat, D.M. Almalki, A. Abushouk, A.M. Saleh, G. Allam, Association between type 1, type 2 cytokines, diabetic autoantibodies and 25-hydroxyvitamin D in children with type 1 diabetes, J. Endocrinol. Invest. 39 (2016) 1425–1434.
[29] P. Kanikarla-Marie, S.K. Jain, Role of hyperketonemia in inducing oxidative stress and cellular damage in cultured hepatocytes and type 1 diabetic rat liver, Cell Physiol. Biochem. 37 (2015) 2160–2170.
[30] R.M. Gomes, L.F. de Paulo, C.P.D.N. Bonarto Panizzon, C.Q. Neves, B.C. Cordeiro, J.N. Zanoni, F.A. Francisco, S. Piovan, P.C. de Freitas Mathias, R. Longhini, J.C.P. de Mello, J.C. de Oliveira, G.R. Pedrino, A.A. da Silva Reis, A.L. Cecchini, M.R. Marçal Natali, Anti-diabetic effects of the ethyl-acetate fraction of Trichilia catigua in streptozotocin-induced type 1 diabetic rats, Cell Physiol. Biochem. 42 (2017) 1087–1097.
[31] R. Murali, S. Srinivasan, N. Ashokkumar, Antihyperglycemic effect of fraxetin on hepatic key enzymes of carbohydrate metabolism in streptozotocin-induced diabetic rats, Biochimie 95 (2013) 1848–1854.
[32] E.G. Giannini, R. Testa, V. Savarino, Liver enzyme alteration: a guide for clinicians, CMAJ 172 (2005) 367–379.
[33] J.A. Usher-Smith, M.J. Thompson, S.J. Sharp, F.M. Walter, Factors associated with the presence of diabetic ketoacidosis at diagnosis of diabetes in children and young adults: a systematic review, BMJ 343 (2011) d4092.
[34] S. Xulu, P.M. Oroma Owira, Naringin ameliorates atherogenic dyslipidemia but not hyperglycemia in rats with type 1 diabetes, Cardiovasc. Pharmacol. 59 (2012) 133–141.
[35] U.J. Jung, M.K. Lee, K.S. Jeong, M.S. Choi, The hypoglycemic effects of hesperidin and naringin are partly mediated by hepatic glucose-regulating enzymes in C57BL/KsJ-db/db mice, J. Nutr. 134 (2004) 2499–2503.
[36] H.H. Mutlu, O.T. Caklili, E. Coskunpinar, Serum concentrations of cyclophilin A in patients with nonalcoholic fatty liver disease, Acta Gastroenterol. Belg. 80 (2017) 3–7.
[37] J.L. Evans, B.A. Maddux, I.D. Goldfine, The molecular basis for oxidative stress-induced insulin resistance, Antioxid. Redox Signal. 7 (2005) 1040–1052.
[38] S.K. Jain, T. Velusamy, J.L. Croad, J.L. Rains, R. Bull, L-cysteine supplementation lowers blood glucose, glycated hemoglobin, CRP, MCP-1, and oxidative stress and inhibits NF-kappa B activation in the livers of Zucker diabetic rats, Free Radic. Biol. Med. 46 (2009) 1633–1668.
[39] T.A. Libermann, D. Baltimore, Activation of interleukin-6 gene expression through the NF-kappa B transcription factor, Mol. Cell. Biol. 10 (1990) 2327–2334.
[40] J.W. Lim, H. Kim, K.H. Kim, Nuclear factor-kappa B regulates cyclooxygenase-2 expression and cell proliferation in human gastric cancer cells, Lab Invest. 81 (2001) 349–360.
[41] S.C. Gupta, C. Sundaram, S. Reuter, B.B. Aggarwal, Inhibiting NF-κB activation by small molecules as a therapeutic strategy, Biochim. Biophys. Acta 1799 (2010) 775–787.
[42] D. Melloul, Role of NF-kappa B in beta-cell death, Biochem. Soc. Trans. 36 (2008) 334–339.