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Naringin prevents bone loss in a rat model of type 1 Diabetes mellitus

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ABSTRACT

The aim of this work was to know whether naringin (NA) could prevent the bone complications in a model of streptozotocin (STZ) induced diabetes. Rats were divided in: 1) controls, 2) STZ-rats, 3) STZ-rats treated with 40 mg NA/kg, and 4) STZ-rats treated with 80 mg NA/kg. BMD and BMC were performed by DEXA. Bone histomorphometry and histology as well as TRAP staining were done in tibia. Osteocalcin (OCN) was determined in bone and serum. Glutathione content and SOD and catalase activities were assayed in bone marrow from femur. The data showed that NA80 increased the BMD and BMC from the long bones of STZ-rats. Both NA40 and NA80 normalized the trabecular number and the trabecular separations. An increase in the number of adipocytes and TRAP(+) cells in tibia from STZ-rats was blocked by NA. NA40 treatment increased the number of OCN(+) cells, but only the NA80 treatment allowed to reach the control values. NA normalized the SOD and catalase activities in bone marrow of femur from STZ-rats. In conclusion, NA avoids alterations in the physical properties and microstructure of bone from STZ-rats probably by stimulation of osteoblastogenesis, inhibition of the osteoclastogenesis and adipogenesis *via* blocking the oxidative stress.

1. Introduction

The impact of Diabetes mellitus (D. m.) on mineral metabolism and bone fragility are not yet totally appreciated [1]. Despite the link between type 1 D.m. and osteoporosis was detected decades ago, this issue has gained attention in recent years [2], maybe in part due to the recognition that bone disorders alter significantly life quality. The risk of fragility fractures is augmented either in type 1 D.m. or type 2 D.m. patients [3]. However, the low bone mineral density (BMD), a risk factor of fragility fracture, is detected in type 1 D.m., whereas in type 2 D.m. the BMD has been found to be normal, low or even high as compared to that of healthy people [4,5]. Insulin treatment is another risk factor for falls and fractures in diabetic patients, as a consequence of increased rate of hypoglycaemic episodes [4]. Certain other medications for the D.m. such as thiazolidinediones and SGTL2 inhibitors [6,8] could also contribute to deteriorating the diabetic bones because of their direct impact on bone and mineral metabolism.

Since histomorphometry evaluates accurately bone turnover, which is rarely undertaken in humans because requires a bone biopsy, animal models become necessary [9]. Streptozotocin (STZ) induced diabetes in mice and rats constitute classical models of type 1 D.m., in which lots of data were obtained increasing the knowledge about the pathophysiology of this disease. Although there is no a conclusive model of bone fragility in D.m., some cellular and molecular mechanisms have been observed. Among them, it has been detected that D.m. is associated with low bone turnover and alteration in bone material properties and in the microstructure, mainly when there are microvascular complications [10]. A decrease in the number and activity of osteoblasts (OB), in the percentage of osteoid area and in the rate of mineral apposition was demonstrated [11]. D.m. also induces lipid accumulation in the bone marrow of long bones, leading to expansion of the bone marrow cavity and reduction in the bone cortical region [12]. With regard to osteoclasts (OC), different studies have reported that their activities in diabetic animals are normal [13], decrease [14] or increase [15]. Maycas et al. [16] have recently found increased apoptosis of osteocytes

Abbreviations: D.m, Diabetes mellitus; BMD, bone mineral density; STZ, streptozotocin; OB, osteoblasts; OC, osteoclasts; NA, naringin; BMC, bone mineral content; DXA, dual energy X-ray absorptiometry; HE, hematoxylin-eosin; TRAP, tartrate-resistant acid phosphatase; OCN, osteocalcin; GSH, glutathione; CAT, catalase; ECLIA, electro-chemiluminescence; SOD, superoxide dismutase.

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and expression of the osteocyte-derived bone formation inhibitor Sost/sclerostin in mice with type 1 D.m. The proposed pathophysiological mechanisms of bone disease in D.m. include hyperglycaemia, oxidative stress, increase in advanced glycation endproducts that alter collagen properties and in the marrow adipogenesis, and release of inflammatory factors, among others [4].

In the last years a great interest has aroused on hypoglycemic agents isolated from natural sources such as bioflavonoids because they are considered to be less toxic, with fewer side effects than those from synthetic sources [17]. Naringin (NA, (4′,5,7-trihydroxy flavonone-7-rhamnoglucoside) is an abundant flavonoid present in citrus fruits with interesting biological and/or pharmacological actions due to its antioxidant, antiapoptotic and anti-inflammatory properties [18]. A protective effect of NA on the activities of the antioxidant enzymes in diabetic animals has been reported [19]. It has also been observed that NA improves ketoacidosis and lipid peroxidation [20] and ameliorates cardiac hypertrophy by reducing oxidative stress and inactivating c-Jun nuclear kinase-1 protein in type 1 Diabetes rat model [21]. So far, there is no information about the effect of NA on bone metabolism in diabetic animals, although osteoanabolic effects of NA have been demonstrated in osteoporotic animals [22,23].

The aim of the present study was to know whether the bone complications of the type 1 D.m. could be prevented by chronic administration of NA in a model of STZ induced diabetes in rats.

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

Male Wistar rats (150–200 g, 2 month old) were maintained at 23–25 °C on a 12 h light-12 h dark cycle, with free access to water and food. Calcium in the diet was around 1%, and vitamin D content was 1000 IU/kg of diet (GEPSA mouse-rat, Pilar, Buenos Aires, Argentina). Two groups of animals were used: 1) controls, and 2) STZ-induced diabetic rats. The second group of animals was injected i.p. with only one dose of STZ (60 mg/kg b.w. dissolved in 0.1 mol/L citrate, pH 4.5 solution), meanwhile the controls were injected with vehicle alone. The blood glucose levels were determined by using a glucometer (AccuCheck; Roche, Germany), after 3 days of STZ or vehicle injection. When blood glucose values exceeded 250 mg/dL and glucose was detected in urine (Multistix, Siemens Medical Solutions Diagnostics, Malvern, USA), the animals were considered diabetic. At 3 days after induction two groups of STZ-treated rats were daily injected subcutaneously with either 40 or 80 mg NA/kg. of b.w. until day 30 and a third group of STZ-treated rats remained without NA treatment. The protocol was approved by the CICUAL (Res.8/15, Commission for Care and Use of Laboratory Animals, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba). The National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) was followed. All efforts were made to minimize the number of animals and their suffering.

2.3. Serum biochemical determinations

Serum glucose (Glicemia enzimática AA), Ca (Ca-Color AA) and P (Fosfatemia UV-AA) were measured employing kits from Wiener Laboratorios S.A.I.C. (Rosario, Argentina), HbA_{1c} was determined by Glycohemoglobin Reagent (Teco Diagnostics, Anaheim, CA, USA) following the manufacturer's operating protocol. Osteocalcin (OCN, N-MID Osteocalcin, Roche Diagnostics) and $25(OH)D_3$ (Vitamin D total, Roche Diagnostics) were determined by electro-chemiluminescence (ECLIA) immunoassays (Modular Analytics E1701, Roche, Mannheim, Germany). Serum insulin was measured by radioimmunoassay (RIA) using an anti-rat insulin antibody (Sigma, St. Louis, Missouri, USA); the minimum detectable concentration was 0.04 ng/mL.

2.4. Dual-energy X-ray absorptiometry

BMD and bone mineral content (BMC) of the left femur and tibia were analyzed by dual energy X-ray absorptiometry (DXA) scanning using a Lunar PIXImus Scanner (Lunar PIXImus2, software version 1.4X) equipped with appropriate software for bone density assessment in laboratory small animals, as reported previously [24]. The distal femoral metaphysis and the proximal tibia were chosen for analysis.

2.5. Histology and TRAP staining

The right tibia from each animal was resected and prepared for histological analysis. After fixation in 4% paraformaldehyde for 48 h at 4 °C, the tibiae were decalcified in 10% EDTA at pH 7 for 30 days, dehydrated, and embedded in paraffin. Sections of 5 μm length were stained with hematoxylin-eosin (HE) and digital microphotographs were obtained to perform the histomorphometric studies and to count the number of adipocytes (Adipocytes/mm^2) using Image Pro Plus 4.5 software (Media Cybernetics, Inc. Rockville, MD 20850 USA). The number of OC per area, TRAP (+) cells/mm^2, was identified by the tartrate-resistant acid phosphatase (TRAP) staining in subchondral bone. All cells with 2 or more nuclei and close to trabeculae were considered osteoclastic cells. After applying TRAP solution (0.1 M acetic acid buffer solution; naphthol AS-MX phosphate; fast red Violet LB salt; N,N-dimethylformamide; Sigma-Aldrich Inc.), the sections were incubated at 37 °C for 30 min. A counterstaining with hematoxylin for 30 s was performed. Images were obtained with a Leica DC 180 Camera (software Leica IM50 Image Manager; Leica, Cambridge, UK). Three independent operators observed the sections and did the counting under the microscope.

2.6. Bone histomorphometry

The following static histomorphometric parameters were measured according to Parfitt et al. [25]: bone volume fraction (BV/TV) (%), the percentage of bone within the total measured area (bone tissue + bone marrow), trabecular number (Tb.N, 1/mm), thickness (Tb.Th, μm) and spacing (Tb.Sp, μm). Digital images of sections from right tibiae were obtained at 10 × magnification with a Leica DC 180 Camera (Leica Microsystems, Mannheim, Germany).

2.7. Inmunohistochemical analysis

Proximal right tibiae were embedded in paraffin and 5-μm thick serial sections were generated and processed according to the streptavidin-biotin peroxidase complex method. Briefly, after deparaffinization, sections were hydrated and incubated for 10 min in 0.5% (v/v) H_2O_2 diluted in methanol to reduce endogenous peroxidase activity. After rinsing with PBS, the slides were incubated with normal bovine serum at 10% (v/v) in PBS for 10 min to avoid non-specific binding of the primary antibody. OCN was localized using an anti-OCN polyclonal antibody produced in rabbits (1:1000 dilution; FL-100, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The antibody was incubated at 37 °C for 1 h. Thereafter, the sections were washed in PBS and incubated with appropriate secondary biotinylated antibodies diluted in

PBS. Half an hour later the sections were incubated with peroxidase-conjugated streptavidin (Histostain-SP Broad Spectrum, Invitrogen, CA, USA) and developed with DAB (Thermo Scientific, Rockford, USA). The same procedure, but without the primary antibody, was used for the control sections. OCN immunoreacted sections were counterstained with hematoxylin. Sections were visualized employing a Leica DM microscope (40X/0.65 N PLAN objectives) and images were obtained with a Leica DC 180 Camera (software Leica IM50 Image Manager; Leica, Cambridge, UK). Three independent operators observed the sections and performed the counting under the microscope.

2.8. Glutathione (GSH) content and catalase (CAT) and SOD activities

Bone marrow was obtained from the right femur and used to determine total GSH content, and CAT (EC 1.11.1.6) and SOD (EC 1.15.1.1) activities. GSH was assayed in the supernatant of bone marrow homogenates by the recycling procedure described by Anderson [26]. Total GSH content is expressed in nmol/mg of protein. CAT activity was assayed in 50 mmol/L potassium phosphate buffer pH 7.4 and 0.3 mol/L H_2O_2 [27]. Mg^{2+}-SOD activity was measured 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 [28]. Enzyme activities are expressed in U/mg of protein.

2.9. Statistical evaluation

All data are expressed as means ± S.E. The results were evaluated by one-way analysis of variance (ANOVA) and the Bonferroni's test as a *post hoc* analysis. 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. Effect of NA on the characteristics of the diabetic rats

Table 1 confirms that after 30 days of STZ injection the rats exhibited weight loss, increase in the serum glucose and in HbA_{1c}, and very low levels of serum insulin, as shown previously [29]. The NA doses employed did not affect these variables. Similarly, serum calcium and phosphorus remained unaltered with the different treatments. The levels of serum calcidiol were highly decreased by STZ injection, and they were kept low under NA treatment. Serum OCN, a biochemical marker of osteogenic activity, was significantly reduced by the STZ injection, remained low after NA40 and almost returned to the control values after NA80 treatment.

3.2. NA improved BMD and BMC of tibia and femur from diabetic rats

As known, the STZ injection deteriorated the BMD and BMC of long bones after 30 days of administration. Daily doses of NA40 did not modify these parameters. However, NA80 improved both BMD and BMC of tibia and femur from STZ rats, reaching the control values (Fig. 1).

3.3. NA avoided the histomorphometric changes caused in rat tibia by the STZ induced diabetes

The insulin deficit provoked alterations in several histomorphometric parameters in the rat tibia. The bone volume (BV/TV), the trabecular thickness (Tb.Th) and the trabecular number were reduced, whereas the trabecular separations (Tb. Sp) were increased. NA40 and NA80 enhanced both the bone volume and the trabecular thickness, but they were not able to reach the control values. However, NA40 and NA80 normalized the trabecular number and the trabecular separations (Table 2).

3.4. NA blocked an increase in the number of adipocytes and TRAP (+) cells in tibia from diabetic rats

After 30 days of STZ injection, the rats showed very high number of adipocytes (Fig. 2) and TRAP (+) cells (Fig. 3) in the subchondral area of right tibiae. NA40 decreased the number of adipocytes as compared to STZ rats, and NA80 reduced much more the number of these cells, which was even lower than that of the control group (Fig. 2). With regard to the TRAP (+) cells, either NA40 or NA80 decreased the number of cells, but the values did not reach the control ones (Fig. 3).

3.5. NA increased the number of OCN (+) cells in tibia from diabetic rats

In the proximal tibia from STZ rats, the number of OCN (+) cells decreased to almost one third as compared to that of control rats. NA40 treatment significantly increased the number of OCN (+) cells, but only the NA80 treatment allowed to reach the control values (Fig. 4).

3.6. NA did not affect the GSH content but normalized both CAT and SOD activities in bone marrow of femur from diabetic rats

GSH content and both CAT and SOD activities were altered in the bone marrow of tibia from diabetic rats. In STZ rats, the GSH content decreased after the diabetic induction, which did not change with NA treatment. In contrast, CAT and SOD activities were augmented by the insulin deficit and returned to the control values after either NA40 or NA80 treatment (Fig. 5).

Table 1
General characteristics from control, diabetic (STZ) and diabetic rats treated with naringin 40 (N40) and 80 (N80) mg/kg b.w.

	C	STZ	STZ + N40	STZ + N80
Body weight (g)	266.80 ± 7.00	179.80 ± 7.90*	179.60 ± 14.10*	185.70 ± 15.10*
Serum glucose (mg/dL)	157.00 ± 4.70	444.60 ± 31.40*	449.00 ± 35.50*	411.70 ± 19.80*
HbA_{1c} (%)	8.00 ± 0.30	11.40 ± 0.60*	11.70 ± 0.30*	11.40 ± 0.40*
Insulin (ng/mL)	1.59 ± 0.03	0.40 ± 0.03*	0.45 ± 0.02*	0.44 ± 0.02*
Serum Ca (mg/dL)	9.92 ± 0.56	9.96 ± 0.44	10.12 ± 0.26	9.55 ± 0.23
Serum P (mg/dL)	5.33 ± 0.22	5.47 ± 0.23	5.59 ± 0.31	5.51 ± 0.24
$25(OH)D_3$ (ng/mL)	10.08 ± 0.76	4.21 ± 0.53*	3.80 ± 0.26*	3.20 ± 0.07*
Osteocalcin (ng/mL)	21.28 ± 1.28	3.78 ± 0.31‡	5.65 ± 0.26‡	17.43 ± 1.33

Values are expressed as means ± S.E. from 8 rats for each experimental condition. C: control; STZ: streptozotocin induced diabetic rats (60 mg/kg b.w.), STZ + N40 and STZ + N80: diabetic rats treated with naringin (40 mg/kg and 80 mg/kg b.w. respectively). Naringin was subcutaneously administered from the third day until the 30th day after STZ injection.*$p < 0.001$ *vs* control; ‡$p < 0.001$ *vs* control and STZ + N80.

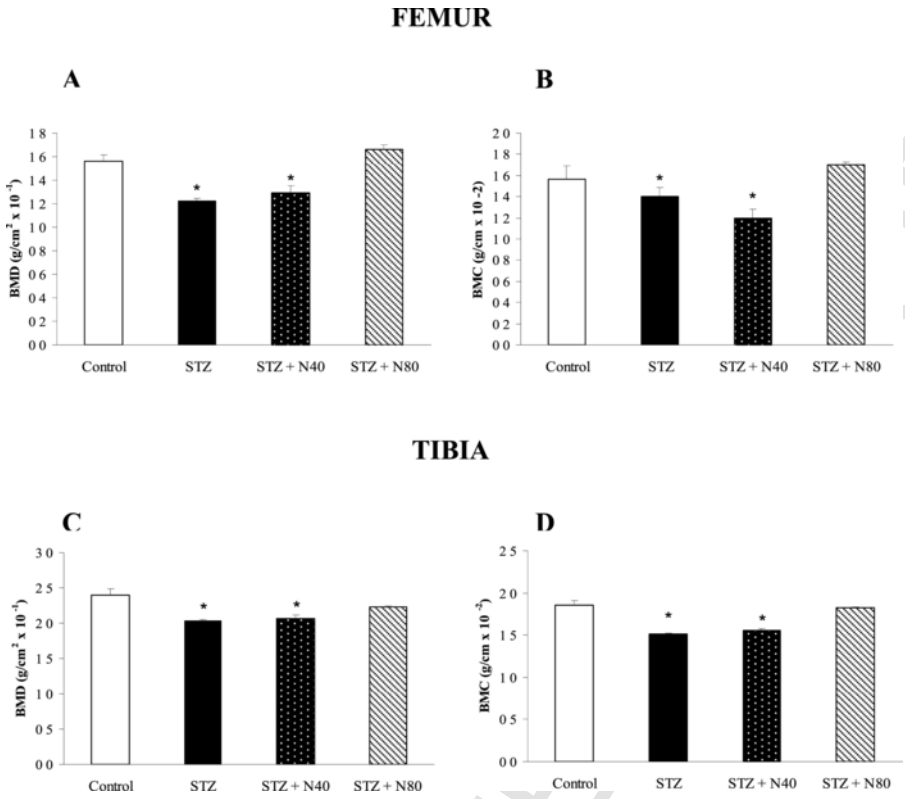

Fig. 1. Bone mineral density (BMD) (**A, C**) and bone mineral content (BMC) (**B, D**) of rat distal femur (**A, B**) and proximal tibia (**C, D**). Values are expressed as means ± S.E. from 7 rats for each experimental condition. STZ: streptozotocin induced diabetic rats (60 mg/kg b.w.), STZ + N40 and STZ + N80: diabetic rats treated with naringin (40 mg/kg and 80 mg/kg b.w. respectively). *$p < 0.001$ *vs* control and STZ + N80.

Table 2
Histomorphometric parameters of proximal tibia from control, diabetic (STZ) and diabetic rats treated with naringin 40 (STZ + N40) and 80 (STZ + N80) mg/kg b.w.

	C	STZ	STZ + N40	STZ + N80
BV/TV (%)	25.02 ± 0.86	12.70 ± 1.28*	20.80 ± 0.76$^{\#}$	18.03 ± 0.72$^{\#}$
Tb. Th (μm)	45.26 ± 1.92	36.59 ± 0.90$^{\#}$	38.54 ± 0.64$^{\#}$	38.64 ± 0.71$^{\#}$
Tb. N (1/mm. 10^{-3})	5.50 ± 0.17	3.30 ± 0.25*	5.40 ± 0.15	5.00 ± 0.09
Tb. Sp (μm)	134.06 ± 5.16	280.20 ± 16.34*	151.31 ± 4.67	169.63 ± 4.88

Values are expressed as means ± S.E. from 8 rats for each experimental condition. C: control; STZ: streptozotocin induced diabetic rats (60 mg/kg b.w.), STZ + N40 and STZ + N80: diabetic rats treated with naringin (40 mg/kg and 80 mg/kg b.w. respectively). BV/TV (%): bone volume, Tb. Th (μm): trabecular thickness, Tb. N (1/mm): trabecular number, Tb. Sp (μm): trabecular separation. *$p < 0.05$ *vs* control, STZ + N40 and STZ + N80; $^{\#}p < 0.05$ *vs* control.

4. Discussion

This study shows that NA is able to inhibit bone alterations caused by the insulin deficiency in STZ rats. This response has been observed after daily injections of NA from the third day until the day 30 post-induction of diabetes. The lack of effect of NA on the body weight, hyperglycemia, hypoinsulinemia and the HbA_{1c} levels indicate that the action of NA on diabetic bone is independent of the systemic metabolic variables related to the glucose metabolism. Similarly, other authors have found no effect of NA on the hyperglycemia in type 1 diabetic rats, but they have detected amelioration of atherogenic dyslipidemia and a significant increase in fasting blood insulin [20,30]. In contrast, the supplementation with NA has been shown to improve hyperglycemia in type 2 diabetic db/db mice via elevated glycolysis and hepatic glycogen content [17].

Our study shows that daily doses of NA80 have a beneficial role in bone mass maintenance since NA improves BMD and BMC either from tibia or femur from diabetic rats. The lack of response with NA40 suggests that this effect is dose dependent. Pang et al. [31] have also demonstrated that a dose of 0.4 mg $NA \cdot g^{-1} \cdot day^{-1}$ for 6 weeks enhances BMD at trabecular-rich bone in OVX mice. Furthermore, in the same animals the authors have shown that NA ameliorates the biomechanical strength of cortical bone. Mandadi et al. [32] have reported that NA at 200 ppm for 2 months improves femoral BMD and strength and BMD of the 5th lumbar spine in young orchidectomized rats. With regard to the trabecular microstructure, we have detected that NA treatment increases bone volume, trabecular thickness, trabecular number, and decreases trabecular separation in comparison with the same parameters from the STZ rats. Similar effects have been observed when OVX mice were treated with NA or NA plus treadmill exercise [33]. However, in normal rats NA does not affect the histomorphological variables of rat bone, which has been observed by us (data not shown) and others [34].

The osteoprotector effect of NA is far from clear. However, there is some evidence that NA promotes the differentiation and proliferation of OB through regulation of BMP-2 mediated by the activation of the PI3K-Akt signal pathway and by the increase in AP-1 binding to the promoter of BMP-2 [35]. NA also enhances the differentiation of bone marrow mesenchymal stem cells into OB [36]. In our study, the increment of serum OCN by NA in diabetic rats is an indication of os-

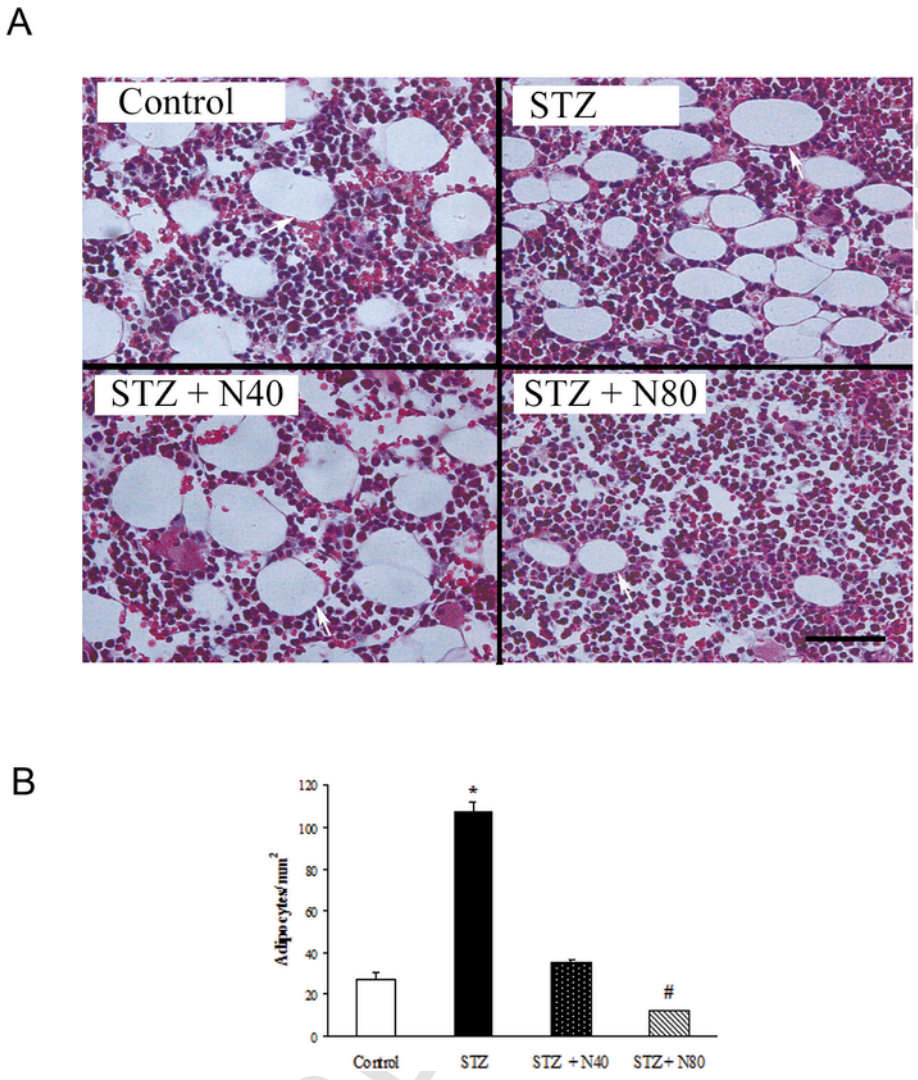

Fig. 2. Analysis of adipocytes number in rat proximal tibia. (**A**) Sections of 5 μm length were stained with hematoxylin-eosin (HE) and digital microphotographs were obtained to count the number of adipocytes (Adipocytes/mm^2) using Image Pro Plus 4.5 software. Adipocytes were indicated with arrows. (**B**) Quantification of the number of adipocytes/mm^2 in proximal tibia. Values are expressed as means ± S.E. from 8 rats for each experimental condition. STZ: streptozotocin induced diabetic rats (60 mg/kg b.w.), STZ + N40 and STZ + N80: diabetic rats treated with naringin (40 mg/kg and 80 mg/kg b.w. respectively). $^*p < 0.001$ *vs* control, STZ + N40 and STZ + N80; $^\#p < 0.01$ *vs* control and STZ + N40.

teogenic activity, which is in consonance with OB differentiation and proliferation. The direct role of NA in enhancing OB differentiation was also verified by immunohistochemistry in bone slices, where the number of OCN(+) cells has been observed to increase by NA treatment in a dose dependent manner. The expression of OCN was also increased by NA in OVX rats that exhibit osteoporosis [37] and in mice with ankylosing spondylitis [38]. OCN is secreted by OB, is stored in the extracellular matrix [39] and is considered a marker of bone formation and bone remodeling [40]. However, OCN is also secreted by other tissues and presents carboxylated and undercarboxylated forms with different functions. New actions and a putative receptor of OCN have been discovered, but the exact mechanisms of functions are far from clear [41].

Our study also shows that NA decreases the number of TRAP(+) bone cells in comparison with that observed in the diabetic condition, which would indicate that NA inhibits osteoclastogenesis. In this address, Ang et al. [42] have reported that NA blocks osteoclastogenesis and bone resorption through inhibition of RANKL-induced NF-kappaB and ERK activation. Although NA decreases the number of TRAP(+) cells in STZ rats, the values do not reach the control ones. Maybe, the dose should be higher or the time of NA administration should be increased.

As previously shown [43], the insulin deficiency increases the number of adipocytes from bone marrow, an effect that is avoided by NA lowering the number of these cells beyond the control values at a dose of NA80. The reason for the last response remains unknown.

Increasing evidence has confirmed that oxidative stress plays a role in the triggering of bone disease associated with D.m. [44]. As expected, the GSH content is decreased in the bone marrow after STZ induced diabetes. GSH is the major intracellular reducing molecule [45]. When intracellular GSH levels are depleted, oxidative stress is triggered. At the doses of our study, NA is not able to increase the bone marrow GSH to reach the control values. In contrast, similar doses of NA increased the GSH content and the ratio GSH/GSSG in sperm of diabetic rats [46]. Whether the effect of NA on GSH synthesis is organ-specific or whether longer times of NA administration could enhance the GSH content in bone are possibilities that need to be investigated. With regard to bone marrow CAT and SOD, the insulin deficiency increases their activities probably as a compensatory mechanism, whereas NA decreases them until returning the values to the normal controls. These data suggest that NA exerts its antioxidant properties,

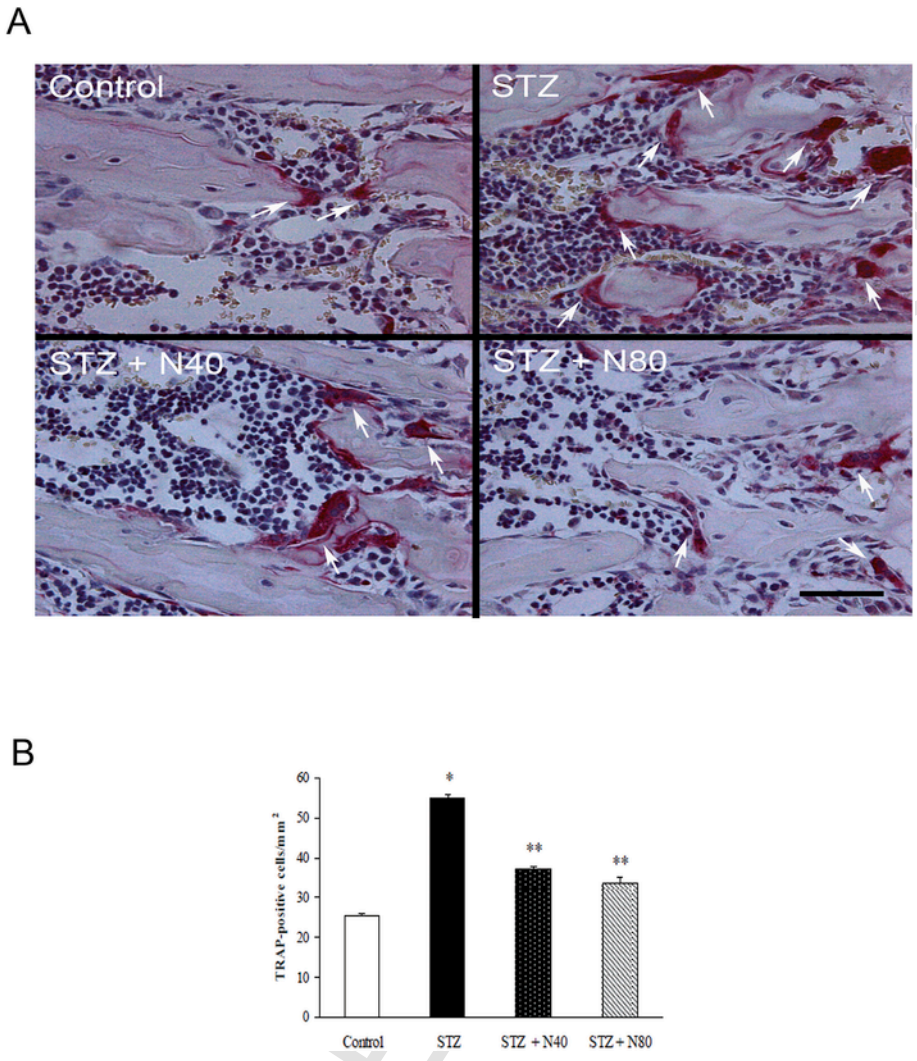

Fig. 3. Counting of OC in rat proximal tibia. (A) Digital microphotographs were obtained to count the number of osteoclasts. TRAP(+) cells with more than two nuclei were counted. (B) Quantification of the number of OC/mm^2 in proximal tibia. Values are expressed as means ± S.E. from 8 rats for each experimental condition STZ: streptozotocin induced diabetic rats (60 mg/kg b.w.), STZ + N40 and STZ + N80: diabetic rats treated with naringin (40 mg/kg and 80 mg/kg b.w. respectively). *$p < 0.001$ *vs* control, STZ + N40 and STZ + N80; **$p < 0.001$ *vs* control.

but the doses and/or the time of administration are not enough to recover totally the bone marrow from the oxidative stress induced by the insulin deficit.

In agreement with some studies in humans and animal models [29,47,48], the levels of calcidiol are lower in STZ rats as compared to those of control rats, which might contribute to increasing inflammation in type 1 diabetes [49]. The vitamin D deficiency is not altered by any dose of NA employed in the present study.

Taken the data together, we can conclude that NA 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 partially, *via* blocking the oxidative stress. Other molecular mechanisms responsible for the bone response under NA administration should be addressed.

Uncited reference

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Acknowledgements

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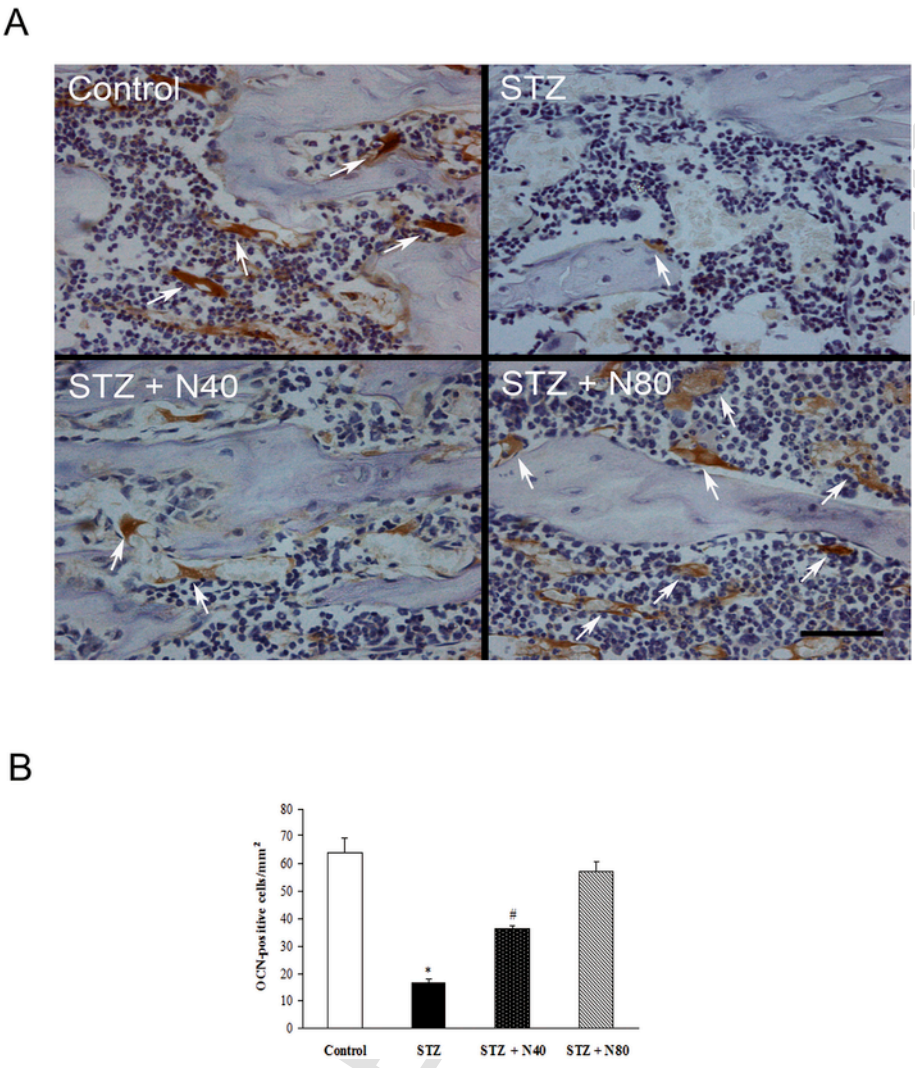

Fig. 4. The number of OCN (+) cells/mm^2 was determined in rat proximal tibia. (A) Digital microphotographs were obtained to count the number of OCN (+) cells. (B) Quantification of the number of OCN(+) cells/mm^2 in proximal tibia. Values are expressed as means ± S.E. from 8 rats for each experimental condition. STZ: streptozotocin induced diabetic rats (60 mg/kg b.w.), STZ + N40 and STZ + N80: diabetic rats treated with NA (40 mg/kg and 80 mg/kg b.w. respectively). *$p < 0.01$ *vs* control, STZ + N40 and STZ + N80; $^{\#}p < 0.01$ *vs* control and STZ + N80.

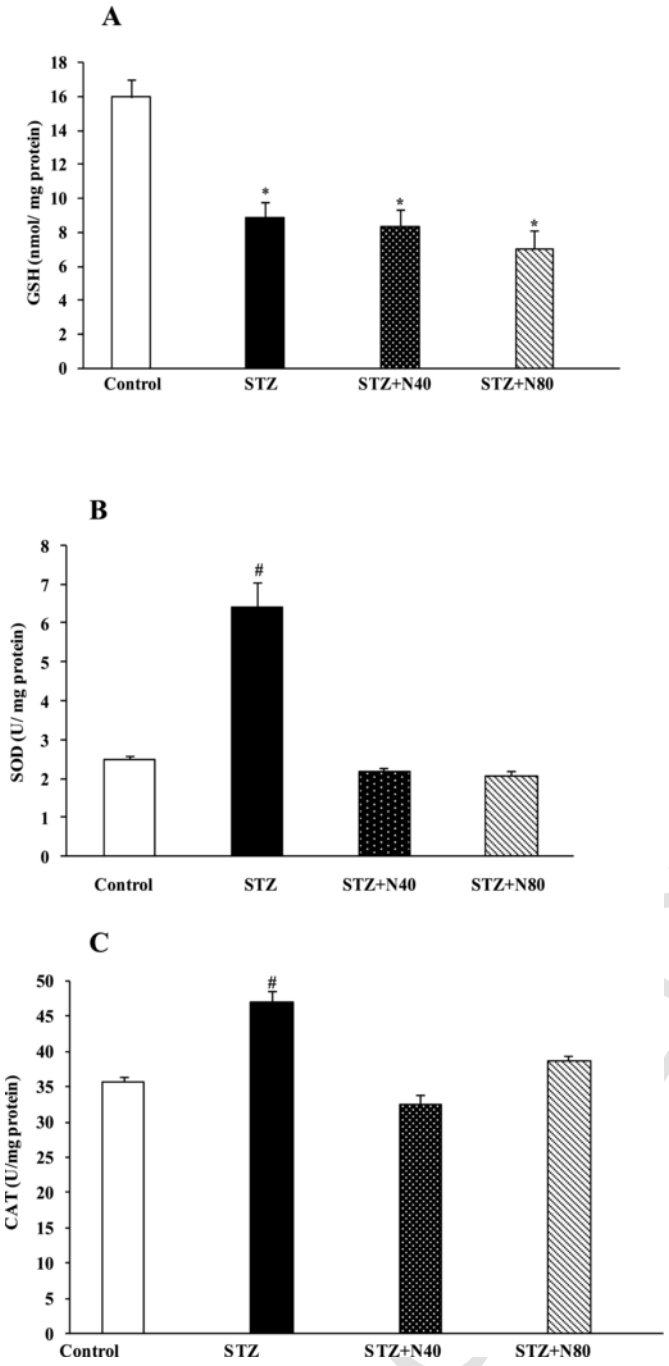

Fig. 5. Determination of total GSH content (**A**) and the activities of SOD (B) and CAT (**C**) in homogenates from bone marrow obtained from control, diabetic rats (STZ, 60 mg/kg b.w.), STZ + N40 and STZ + N80 (diabetic rats treated with naringin (40 mg/kg and 80 mg/kg b.w., respectively). Data represent means ± S.E. from 7 rats for each experimental condition. *$p < 0.01$ *vs* control; #$p < 0.001$ *vs* control, STZ + N40 and STZ + N80.

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