

The effects of natural nano-sized clinoptilolite and *Nigella sativa* supplementation on serum bone markers in diabetic rats

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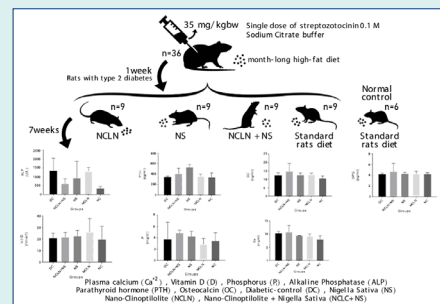
Abstract

Introduction: Many studies confirm that diabetes mellitus is associated with higher risks of bone fracture. The beneficial effects of *Nigella sativa* (NS) and clinoptilolite in preventing/reducing some diabetes-related disorders have been shown. This study was conducted to examine the effects of separate and concurrent supplementation of natural nano-sized clinoptilolite (NCLN) and NS on serum bone markers in rats with type 2 diabetes.

Methods: A total of 42 (case=36 and control=6) adult male Wistar rats were divided into 2 groups: diabetic and non-diabetic. An oral glucose tolerance test and a homeostatic model assessment of insulin resistance (HOMA-IR) test were conducted to confirm diabetes. Then, the diabetic group was divided into 4 subgroups: [1] control (n=9), [2] NS 1%/food (n=9), [3] NCLN 2%/food (n=9), [4] NS 1%/food + NCLN 2%/food (n=9). After 7 weeks, serum levels of bone markers were determined using ELISA kits.

Results: Analysis showed that serum levels of alkaline phosphatase (ALP) in the NCLN group (1318.6 ± 217.5 U/L) was significantly ($P < 0.05$) higher than other intervened groups. On the other hand, serum levels of calcium in NCLN+NS group (10.8 ± 2.6 mg/dL) were higher ($P = 0.027$) compared to all other study groups. However, rats in the NS group had higher (535.8 ± 49.3 pg/mL) PTH ($P < 0.0001$) compared to other supplementation groups. There were no significant differences in vitamin D and osteoprotegerin.

Conclusion: The results of the current study suggest that bone mineralization may be affected by concurrent use of NS and NCLN through influencing calcium circulation. Moreover, dietary NS administration is strongly related to an augmented level of PTH.



Introduction

Diabetes is known as an increasingly prevalent disease and is highly associated with morbidity and mortality.¹ According to the estimations of the International Diabetes Federation, the number of patients suffering from diabetes was 387 million in 2014, and will reach 592 million by 2035.² Type 2 diabetes mellitus (T2DM) is a progressive, multifactorial disease characterized by hyperglycemia due to defects in insulin secretion or insulin action (which results in insulin resistance), or both. Inadequate glycemic control and long-term hyperglycemia both play important

roles in the development of diabetic complications and lead to a wide range of diseases including neuropathy, nephropathy, retinopathy, and macrovascular diseases.^{1,3,4} Moreover, T2DM patients suffer from various bone-related disorders such as osteoporosis and fractures.⁵ Bones are influenced by diabetes through multiple mechanisms, such as insulin resistance, insulin deficiency, hyperglycemia, and atherosclerosis.⁶ Bone turnover is affected by hyperglycemia, and decreases in bone markers in blood serums of patients with diabetes. Diabetes is associated with higher risks of fracture and decreased



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bone mineral density.⁷

Regarding numerous diabetes-related disorders and mortalities, progressive investigations have been conducted to find more effective drugs to reduce the social burden of diabetes. In previous studies, various supplementations have been done for improvement of some diabetes-related parameters.⁸⁻¹¹ Zeolites are highly crystalline and porous minerals which have been exploited in environmental, industries, agricultural and biomedical technologies.^{12,13} The protective effects of zeolites A (kind of syntactic zeolites) have been documented in previous studies, through an increase in alkaline phosphatase (ALP) activity, proliferation and cell differentiation in osteoblasts.^{14,15} Moreover, significant associations have been shown in animal studies previously between diabetes and *clinoptilolite*, a natural zeolite supplementation and calcium concentration.¹⁶ In our previous study nano-sized *clinoptilolite* (NCLN) decreased glucose levels in type 1 diabetes.^{17,18}

Beside zeolites, *Nigella sativa* (NS) has been demonstrated as another recommended herbal treatment in both diabetes and bone disorders.^{19,20} NS, which belongs to the Ranunculaceae family, is an annual plant which is grown in many countries.²¹ *Thymoquinone* (TQ) is the most abundant and most active constituent of NS.¹⁹ The associations between NS and bone metabolism, formation, as well as in the treatment of bone complications, have been shown in earlier investigations. Moreover, the anti-hyperglycemic effects of TQ have been demonstrated in previous animal studies.²²⁻²⁴

Considering the evidence of the beneficial effects of both of NCLN and NS on diabetes²⁵⁻²⁸ and bone disorders, as well as the potential side effects of high-dose supplementation when used separately (which could be prevented by concurrent use), and because of the probable synergistic effects of the two, this study aimed to test their effects on serum bone markers in T2DM rats.

Materials and Methods

Animals

A total of 42 adult male Wistar rats, including 36 diabetic and 6 healthy ones (normal control=NC), weighing more than 250 g and having a mean age of 5 to 6 months, were obtained from the animal breeding center of Tabriz University of Medical Sciences. Diabetic rats were divided into 4 subgroups, each of them including 9 rats, as follows: (1) received only NCLN diet, (2) received only NS diet, (3) received both NS and NCLN diet, and (4) received standard rat's diet (as diabetic control=DC). Rats were chosen according to the following defined inclusion criteria: same age (5-6 month), sex (male) and race (Wistar), fasting blood glucose (BG) more than 250 mg/dL, and proven diabetic through oral glucose tolerance test (OGTT) after 12 hours fasting. Moreover, any rats which died before the end of the study were excluded (15 rats). The rats were kept under controlled conditions of

temperature (23-25°C), humidity (30% to 50%), and a 12-h light/dark cycle. Rats were given unrestricted access to food and water through the experiment.

Preparation of therapeutic diets

Natural zeolite (Afrazand Co., Tehran, Iran) is a sodium/potassium *clinoptilolite* with a particle size of approximately 5 µm. The NCLN particle was produced from *clinoptilolite* by glow discharge plasma method which is a novel Fe-Impregnated Nanocatalyst for the Heterogeneous Fenton Process. Glow discharge plasma was used to convert microparticles of CLN to nanorods (in the Research Institute for Applied Physics and Astronomy at University of Tabriz). The physicochemical properties of the prepared NCLN (size and morphology) and the procedure of synthesis are thoroughly presented in Khataee et al, 2013 published data.²⁹ Particle analysis of the NCLN as compared to *clinoptilolite* is presented in our previously published article.^{26,29} Seeds of NS was obtained from Tabriz city's local market. A grinder was used to powder NCLN. NS and NCLN powders were prescribed in the pelleted form: 1,000 mg/kg BW and 2% respectively, for 7 weeks.

Induction of type 2 diabetes in rats

T2DM was induced by a month-long high-fat diet (HFD) (48% carbohydrate, 32% fat and 20% protein), followed by an intraperitoneal injection of 35 mg/kg BW single dose Streptozotocin 0.1 M sodium citrate buffer, at pH 4.5. One week after injection, BG levels were obtained from the orbital sinus (1-2 drops) to determine a diagnosis of diabetes. An Accu-Chek glucometer (Roche, Germany) was used to determine BG. Rats with BG levels of ≥ 250 mg/dL were classified as diabetic. Moreover, we excluded rats with BG levels of ≤ 250 mg/dL.

Oral glucose tolerance test and homeostatic model assessment of insulin resistance (HOMA-IR)

An OGTT with 12 hour of fasting was conducted to ensure T2DM induction. Then, a 20% glucose (2 g/kg BW) solution was given to the animals. Blood samples were obtained from the animal's tail to measure BG and insulin levels, after 0, 30, 60 and 120 minutes. After 7 weeks, at the end of treatment, HOMA-IR was used for estimating insulin resistance as follows: Plasma glucose (mg/dL) \times fasting plasma insulin (µU/L) divided by 405.

Collection of blood samples and estimation of biochemical parameters

Serum osteocalcin levels, blood vitamin D levels, parathormone (PTH), and osteoprotegerin (OPG) levels were determined using ELISA kits (East Biopharm Co, China). Moreover, ALP and calcium concentrations were determined using ParsAzmun kits and phosphor concentrations were determined using a ZiestChem Diagnostics kit.

Statistical Analysis

Descriptive statistics such as mean and standard deviation (SD) were used to summarize continuous variables. For evaluation, a normal distribution of numeric variable descriptive measures (skewness and kurtosis) were used, with values outside of the range (1.5 and 2 showing a deviation from normality, respectively). To compare the presence of significant differences in variables between different groups of rats, one-way ANOVA was used, followed by the Tukey post hoc test. Data analysis was conducted by using Prism GraphPad 6 software. $P < 0.05$ was considered significant.

Results

Comparison of blood parameters between different groups of rats

Mean (SD) for plasma glucose levels after consumption of HFD and injection of Streptozotocin but before interventions were as follows: DC: 458.66 (107.96); NCLN+NS: 477.33 (84.24); NS: 429.66 (63.56); NCLN: 532.57 (94.71). The levels of FBG after interventions are presented in our another research article which is in press.³⁰ Table 1 shows the effect of the prescribed diets on blood mineral concentrations. After 7 weeks of intervention, plasma Ca levels in the DC group was significantly higher than NC ($P=0.025$). Post-Hoc tests indicated that Ca levels

in the NC group were lower than in the DC ($P=0.049$) and NCLN+NS groups ($P=0.027$).

Comparison of bone parameters between different groups of rats

The effects of dietary interventions after 7 weeks of intervention are shown in Tables 1 and 2. The analysis demonstrates significant differences in the ALP plasma levels between diabetic and control rats ($P=0.012$). Tukey post-hoc test confirms that the ALP serum levels were higher in the NCLN intake group than in NC rats ($P=0.026$). Serum levels of osteocalcin in NCLN+NS group were 33.3% higher than NC group. Serum levels of mineral and bone markers were significantly ($P < 0.05$) different between diabetic and non-diabetic groups. There was a higher percentage of vitamin D levels in the NCLN rats, compared to others. NCLN+NS group showed a 40% and 58% decrease of ALP than the NS and NCLN groups, respectively.

Comparison of PTH, vitamin D and osteoprotegerin serum levels between different groups of rats

As presented in Table 2, there were significant differences between diabetic and NC groups in serum levels of PTH. The NS group had a higher PTH than all the diabetic (DC, NCLN, and NS+NCLN), and NC groups. There were no

Table 1. Mean (SD) of vitamin D, calcium and phosphorus levels in rats after interventions

		DC n=6	NCLN+NS n=5	NS n=5	NCLN n=7	NC n=6	P value
Vit D (nmol/L)	Mean (SD)	20.9 (4.4)	21.8 (4.2)	22.6 (5.17)	26 (11.9)	19.8 (11.5)	0.72
	95% CI	16.2-25.5	15.8-26.4	16.1-29	15-37	7.6-31.9	
Ca (mg/dL)	Mean (SD)	10.4 (0.74**)	10.8 (2.6)	9.5 (0.1)	9.3 (0.8)	8.1 (1.3**)	0.025*
	95% CI	9.6-11.2	7.5-14.1	9.3-9.8	8.4-10.1	6.7-9.5	
P (mg/dL)	Mean (SD)	3.8 (3)	4.9 (0.5)	4.3 (0.9)	2.9 (1)	3.5 (1.4)	0.36
	95% CI	0.7-7	4.2-5.5	3.1-5.5	1.9-3.9	2-5	

Standard Deviation (SD), Plasma calcium (Ca^{2+}), vitamin D (D), Phosphorus (P), Diabetic-control group (DC), Normal Control group (NC), Nigella sativa (NS), Nano- Clinoptilolite (NCLN), Nigella Sativa+ Nano- Clinoptilolite (NCLN+NS).

ANOVA test (* P value < 0.05 between group) followed by Post-Hoc tests: ** P value < 0.05 compared to group DC & NC.

Table 2. Mean (SD) of osteocalcin, alkaline phosphatase and osteoprotegerin and parathyroid hormone levels in rats after interventions

		DC n=6	NCLN+NS n=5	NS n=5	NCLN n=7	NC n=6	P value
Bone Biomarker							
OC (ng/mL)	Mean (SD)	12.6 (1.4)	14.8 (4.7**)	12.7 (1.2)	13 (0.8)	10.7 (1.4**)	0.082
	95% CI	11.1-14.1	8.9-20.8	11.2-14.2	12.3-13.8	9.1-12.2	
ALP (U/L)	Mean (SD)	1347.2 (711**)	618.4 (279.9)	925.3 (946.9)	1318.6 (217.5)	361.7 (85.5**)	0.012*
	95% CI	601.7-2093.3	270.8-966	250.4-2101.1	1117.4-1519.8	271.8-451.5	
OPG (pg/mL)	Mean (SD)	4.2 (0.2)	4.7 (1.2)	4.3 (0.3)	4.3 (0.5)	4.2 (0.3)	0.64
	95% CI	3.9-4.4	3.2-6.2	3.9-4.7	3.7-4.8	3.9-4.5	
Hormonal							
PTH (pg/mL)	Mean (SD)	349.2 (17.4)	405.6 (111**)	535.8 (49.3**)	360.5 (40.7**)	343 (84**)	$< 0.0001^*$
	95% CI	330.8-367.5	267.7-543.5	474.5-597	322.8-398.2	254.8-431.2	

Standard Deviation (SD), Alkaline phosphatase (ALP), Parathyroid hormone (PTH), osteoprotegerin (OPG), osteocalcin (OC), Diabetic-control group (DC), Normal Control group (NC), Nigella Sativa (NS), Nano-Clinoptilolite (NCLN), Nigella Sativa+ Nano- Clinoptilolite (NCLN+NS).

ANOVA test (* P value < 0.05 between group) followed by post hoc tests. ** P value < 0.05 .

significant differences in vitamin D and osteoprotegerin levels among groups.

Discussion

This study investigated the effects of concurrent and separate dietary supplementation of NS and NCLN on bone markers and mineral levels in T2DM rats. ALP and calcium levels increased following diabetic induction. Inconsistent with our study, Celebi et al³¹ reported a dose-dependent increasing trend of ALP and calcium levels with rises in CLN intake in laying hens. Additionally, other investigations conducted by Bachman et al³² and Prvulovic et al³³ found no significant relationship between CLN and ALP levels among crossbred beef heifers and pigs, respectively. Recent investigations attributed ALP and osteoblastic activity to CLN. Maxwell D.B. et al. showed a positive relationship between ALP and blood sugar,³⁴ thus higher levels of ALP among diabetic groups than NC were expected in the current study. The additive effect of CLN on higher levels of insulins³⁵ was expected to decrease glucose levels in the diabetic-control group more than the normal-control.

Serum osteocalcin levels were higher in the NS+NCLN group compared to healthy rats. The inverse association between osteocalcin (OC) and glucose levels has been reported in previous studies.³¹ Different forms of OC seem to play a leading role in promoting insulin synthesis, lowering blood sugar and improving insulin resistance.^{32,33} Masahiro et al. found that the presence of diabetes is associated with lower OC levels.³⁴ The higher levels of OC found in the NCLN group in this study were in contrast to Jianwen et al's findings. In both studies, rats were fed HFD, but their results showed lower levels of OC in spite of HFD.³⁵

In this study, it was also observed that the NS group had the highest PTH, compared to the other 4 groups. PTH is a hormone with 84-amino acid peptide, which acts as a mediator of bone remodeling and is a vital regulator of calcium ion homeostasis.³⁶ Higher PTH in the NS group compared to NC was in contrast with findings of Masahiro et al's study, in which the incidence of diabetes was related to lower PTH. The supportive effects of PTH levels on bone anabolism through IGF signaling has been proposed.³⁶ Further study is needed to clarify this effect. It was also found that the NS+NCLN group had lower PTH levels compared to the NS group, which raises the question of whether NCLN reduces PTH levels.

The present study highlights the increasing effects of NCLN+NS supplementation on serum calcium levels. In a review study, Ahmad et al³⁷ reported that NS acts as a calcium source. Nehar et al³⁸ confirmed improvements in insulin levels through increasing Beta-cell differentiation and insulin sensitivity by NS intake among diabetic pigs, thus enhancing calcium re-absorption from nephron tubes,³⁹ which as a result, elevated calcium levels in blood circulation. In addition, Berto et al⁴⁰ indicated a direct

Research Highlights

What is the current knowledge?

✓ Many studies have confirmed that diabetes mellitus is associated with higher risks of bone fracture.

What is new here?

✓ The beneficial effects of *Nigella sativa* and clinoptilolite in preventing/reducing some diabetes-related disorders were shown.

✓ The combined use of *N. sativa* and nano-sized clinoptilolite (NCLN) may improve bone mineralization.

relationship between NCLN and calcium concentrations. According to the mechanisms of combined NS and NCLN with calcium levels in circulation, it seems that NS + NCLN intake leads to improved bone mineralization.

Since the administration of HFD separately may affect some parameters in our study, such as blood OC level (documented in previous studies, as mentioned above), the confounding effects of HFD-induced diabetes were the main limitation of the current study. Moreover, another limitation was the inadequate duration of the study to determine the effects of diabetes on serum bone markers. As far as this research team is aware, the strength of this study is that it is the first study that examines the impacts of NS either separately or combined with NCLN on bone markers in diabetic rats.

Conclusion

In conclusion, the current paper suggests that NS with NCLN may be related to bone mineralization improvement through influencing calcium circulation, and may also increase blood levels of osteocalcin, consequently effecting BG and metabolism levels. Further research may be needed to clarify this exact mechanism. Moreover, dietary NS administration is highly related to augmented PTH levels.

Founding sources

This research study was financially supported by Tabriz University of Medical Sciences.

Ethical statement

The study protocol was approved by the bioethics committee of Tabriz University of Medical Sciences (ethical code number: IR.TBZMED.REC.1395.30).

Conflict of interests

The authors declare that they have no conflict of interest.

Authors' contribution

YS has contributed in designing the study, handling the data, analyzing the data and preparation of the draft. SK has contributed in research idea and preparation of nano-sized clinoptilolite (NCLN). MM has contributed in handling the data. MAJ has contributed in analyzing the data. ATE has contributed in research idea, designing the study and preparation of the draft. EB has contributed in handling the data. HO has

contributed in research idea and handling the data.

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