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Original article

Effects of separate and concurrent supplementation of Nano-sized clinoptilolite and Nigella sativa on oxidative stress, anti-oxidative parameters and body weight in rats with type 2 diabetes

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ABSTRACT

Objectives: The objective of this study was to investigate the effects of separate and concurrent supplementation of natural nano-sized clinoptilolite (NCLN) and Nigella sativa (NS) on oxidative stress (OS), anti-oxidative parameters and body weight (BW) in high-fat-diet (HFD)/streptozotocin (STZ)-induced diabetic rats.

Methods: In this experimental study, 42 male Wistar rats were divided into diabetic (n = 36) and non-diabetic (n = 6) groups. The diabetic group (DG) was fed with a HFD for one month, then injected with intra-peritoneal single dose STZ (35 mg/kg BW). The DG was divided into 4 subgroups: [1] control (DC), [2] NS 1%/food, [3] NCLN 2%/food, [4] NS 1%/food + NCLN 2%/food. At the end of the 7th week, malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPX) levels and total antioxidant capacity (TAC) were measured.

Results: The MDA level was decreased in the NCLN ($p = 0.011$) and NCLN + NS ($p = 0.007$) groups compared to the DC group. The GPX level increased in the NS and NCLN groups compared to the DC group ($p = 0.014$ and $p = 0.034$). In addition, the level of TAC demonstrated increase in the untreated DG and NS groups, as compared to the normal control (NC) group ($p_{DC} = 0.031$ and $p_{NS} = 0.024$). Moreover, in the NS + NCLN group, the level of SOD decreased in comparison to the NS and NCLN groups ($p < 0.01$). At the end of the 7th week, BW decreased in the diabetic subgroups in comparison to the NC group. Treatment with NS and/or NS + NCLN insignificantly prevented severe weight loss in the fifth week of the treatment.

Conclusions: According to results, separate supplementation of NS and NCLN was more beneficent on anti-oxidative parameters than concurrent supplementation of NS and NCLN.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a multifactorial and widespread metabolic disorder characterized by relative defects of pancreatic β-cells in secreting insulin in response to glucose and/or insulin resistance in target tissues, leading to impaired glucose regulation [1,2]. Hyperglycemia, polydipsia, polyuria, polyphagia and glycosuria are the main symptoms of diabetes [3].

According to the International Diabetes Federation statistics, diabetes currently affects 246 million people worldwide; this number is expected to reach to 380 million in 2025 [4]. The most common complications associated with this devastating disease include ocular, renal, and cardiovascular disorders caused by hyperglycemia and/or long-term macronutrients metabolism disturbances [5].

Reactive oxygen species (ROS) are generated in small amounts by the mitochondria during physiological processes. Excessive production of free radicals, particularly ROS can result from chronic hyperglycemia [6]. The aggregating role of ROS in Streptozotocin (STZ)-induced tissues damage has been previously reported in diabetic animal models [7]. On the other hand, oxidative stress (OS) has been shown to

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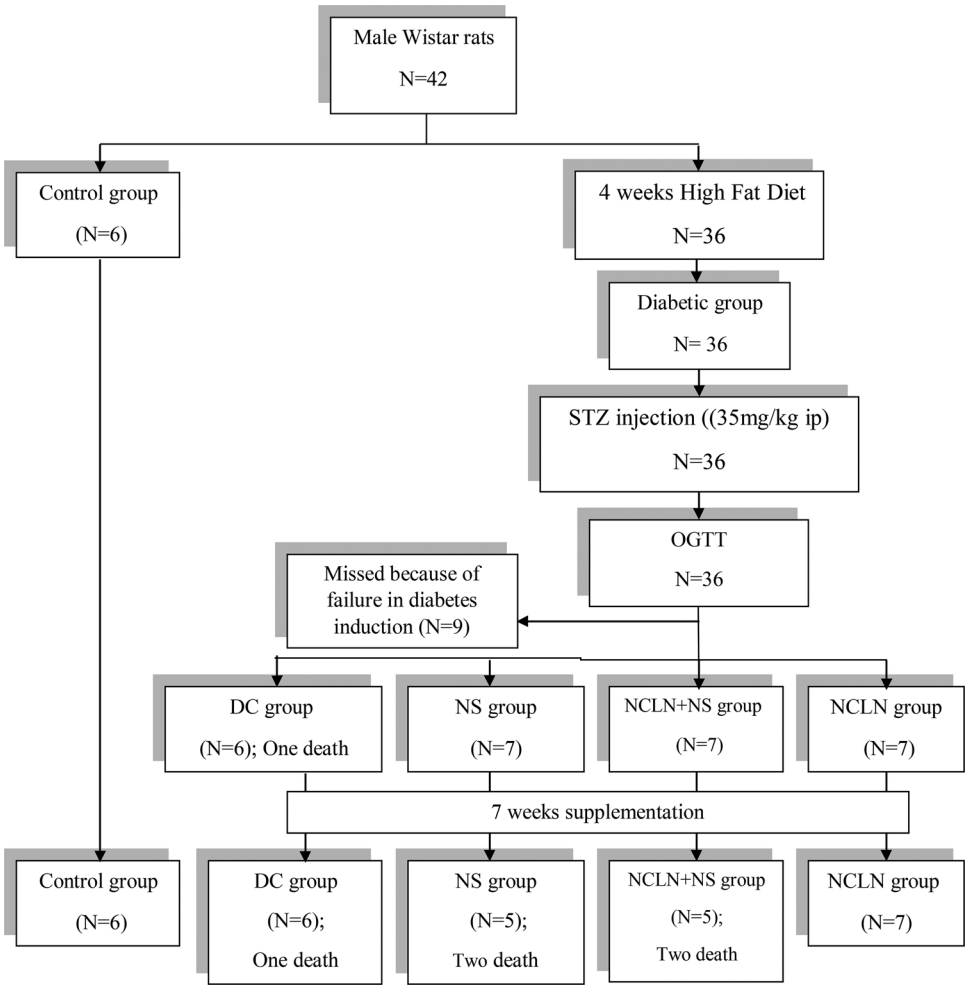

Fig. 1. Diagram of study's steps.

exacerbate T2DM, increase oxidative damage of lipids and DNA, and impair anti-oxidative defense systems in diabetic subjects [8]. Patients with diabetes have higher plasma malondialdehyde (MDA) concentration, one of the end products of lipid peroxidation, but lower plasma total antioxidant capacity (TAC) level and erythrocyte superoxide dismutase (SOD) activity [9,10].

Due to failure to achieve optimum glycemic control by the existing therapeutic strategies such as oral anti-diabetic agents and despite extensive research in treating T2DM, achieving more effective treatments with fewer side effects is necessary for diabetes mellitus management [11]. Because of the unfavorable side effects of long-term or high dose consumption of some antioxidants, researchers are seeking for harmless antioxidants [12]. Two known substances that have recently attracted the attention of many researchers in treating diabetes and several other diseases are zeolite and Nigella sativa (NS) [13].

Nigella sativa is a native herbaceous plant relating to the Ranunculaceae or Buttercup family, and has been used as a food seasoning and a natural remedy for some complications and diseases. Thymoquinone (TQ), the major components of NS, has antioxidant properties [13]. Various pharmacological activities of NS, including anti-diabetic, anti-oxidative [8], PPAR-γ modulatory, cardio-protective [14], anti-inflammatory [15] and anti-cancer [16] effects have been reported. Moreover, NS has been used as a liver tonic, digestive, anti-diarrheal, appetite stimulant, and immune system booster [17]. In addition, increasing evidence supports the usage of TQ and/or NS oil in reducing triglyceride (TG), and cholesterol levels, lipid peroxidation, and increasing the activity of SOD, and glutathione peroxidase (GPX) [18]. Indeed, TQ and NS oil, inhibit lipid peroxidation in liposomes, through non-enzymatic process [19].

Clinoptilolites (CLNs), as the most abundant natural zeolites are hydrated natural or synthetic microporous crystals comprised of AlO_4 and SiO_4 tetrahedral constructions linked through common oxygen atoms with high specific surface area. Because of its caution exchange, adsorption, and catalytic characteristics, it has been widely used in industry and agriculture for feeding animals, filter dialysis machines and remove dissolved ammonia from drinking water. Additionally, CLN has been reported to exert immune-enhancing, anti-carcinogenic and anti-oxidative effects in *in vitro* and *in vivo* studies [20–23]. Also, it has been shown that silica supplementation (the main ingredient of zeolite) in rats injected with STZ, prevents the development of diabetes and preserves pancreatic beta cells from damage [24]. The ability of CLN in decreasing free radicals and lipid peroxidation level as well as increasing total antioxidant capacity (TAC) in Serum is attributed to its anti-oxidative characteristics [23]. No toxic effects of CLN have been documented [22]. Nano-sized clinoptilolite (NCLN), which is produced from CLN by glow discharge plasma method, has been shown to increase the absorption of glucose and other substances [25].

Concurrent supplementation of NCLN and NS may cause synergistic effects in decreasing OS and improving anti-oxidative capacity while preventing allergic reaction and other side effects on vital organs. Considering our earlier promising evidence on the effectiveness of NCLN + NS on BG in T2DM, lack of studies assessing the effect of NCLN on OS and the uncertainty of the effective type and dose of NS in T2DM, this study aimed to investigate the synergistic effects of these two materials on OS, antioxidant parameters and body weight (BW) in T2DM rats.

Table 1
Elemental composition of clinoptilolite and nano-sized clinoptilolite.
Adapted with permission from Table 2 in "Khataee, A., Bozorg, S., Khorram, S., Fathinia, M., Hanifehpour, Y., & Joo, S. W. (2013). Conversion of natural clinoptilolite microparticles to nanorods by glow discharge plasma: a novel Fe-impregnated nanocatalyst for the heterogeneous Fenton process. *Industrial & Engineering Chemistry Research*, *52*(51), 18225–18233."

	Weight (%)				mole/ratio		
	Na	Al	Si	K	Si/Al	Na/Al	K/Al
CLN	3.58	7.07	60.33	0.72	8.28	1.25	0.15
NCLN	8.86	4.81	44.27	10.94	8.88	4.52	3.23

CLN = clinoptilolite; NCLN = nano-sized clinoptilolite; Na = Sodium; Al = Aluminium; Si = Silicon; K = Potassium.

2. Methods and materials

2.1. Chemicals

Streptozotocin (STZ, Sigma Chemicals, and St. Louis, MO, USA), Diethyl ether and other solvents and buffers (Merck, Germany C) were used in this project.

2.2. Animals

A total of 36 intervention and 6 control male Wistar rats aged 5–6 months weighing 250 > g, were included in this study; the animals were obtained from the Animal Center of Tabriz University of Medical Sciences. All animals were fed with standard *ad-libitum* diet and normal drinking water and kept in cages for 4–5 days in order to acclimate with the temperature, humidity, and light of the environment (cycle dark/light 12 h). A month after administrating the high-fat diet and subsequent STZ injection, the oral glucose tolerance test (OGTT) was conducted for the certainty of diabetes induction. At the end of the study (7th week), OS and antioxidant parameters were assessed by obtaining 5 ml blood sample from animal's heart (Fig. 1). Serum MDA level was evaluated with the reaction of thiobarbituric acid (TBA) as a TBA reactive substance (TBARS) in order to produce a pink colored complex. Then, the fluorescence intensity of this complex was measured at 547 nm with 525 nm excitation rate using a spectrofluorimeter (Kontron, model SFM 25A, Italy). Measurement of TAS in Serum and SOD and GPX in heparinized blood was performed by colorimetric method using commercial kits (TAS: RANDOX kits, SOD: RANSOD kits and GPX: RANSEL kits; RANDOX Laboratory, UK). All moral issues regarding the maintenance and operation of the animals were respected, and ethical approval was obtained from the ethics committee of Tabriz University of Medical Sciences.

2.3. Grouping of animals

The tested (diabetic) rats were divided into four groups of 9, based on the diet they received: 1) only NS diet, 2) only NCLN diet, 3) both NS and NCLN diet, 4) standard rat diet (diabetic control = DC). The healthy group (n = 6) also received a standard rat diet (normal control = NC).

2.4. Induction of T2DM in rats

For inducing T2DM, the animals were administered with a high fat diet (HFD) (48% carbohydrate, 32% of energy from fat, 20% protein as a percentage of total kcal) *ad libitum* for one month followed by intraperitoneal injection of a single dose STZ (35 mg/kg BW) in 0.1 M sodium citrate buffer (pH = 4.5) [26]. The composition of normal pellet diet (NPD) and HFD are described in Table 2. For diagnosing diabetes, one week after the injection, BG levels were assessed from the blood sample obtained from the orbital sinus (1–2 drops). BG levels were determined using an Accu-Chek glucometer (Roche, Germany) and rats with high BG of 250 mg/dL were considered diabetic and selected for examination.

Table 2
Elemental composition of NPD and HFD regimens.

	NPD (%)		HFD (%)
Crude Protein	22.5–23.5%	Powdered NPD	50
Crude Fat	3.5–4.5	Rump oil	25
Crude Fiber	4–5	Sucrose	12
Lysine	1.15–1.2	Chickpea flour	1
Methionine	0.33–0.37	Wheat flour	10
Methionine + cysteine	0.63–0.65	Cholice acid	1
Threonine	0.72–0.75	Cholesterol	1
Tryptophan	0.25–0.32		
Ash	Up to 10		
Calcium	0.95–1		
Phosphorus	0.70		
Salt	0.5–0.55		

NPD = normal pellet diet; HFD = high fat diet.

2.5. Oral glucose tolerance test (OGTT)

For the certainty of T2DM, OGTT was also carried out. For this purpose, a solution containing 20% glucose (2 g/kg BW) was prescribed by oral gavage. After 0, 30, 60 and 120 min, blood samples were taken from the orbital sinus for measuring BG and insulin concentration.

2.6. Preparation of therapeutic diets

The NS seeds (Tabriz, Iran) and CLN (Afrazand Co., Tehran, Iran) were purchased from local markets. The NCLN particle was produced from CLN by glow discharge plasma method which is a novel Fe-Impregnated Nanocatalyst, essential for the Heterogeneous Fenton Process. Particle analysis of the NCLN in comparison to CLN is presented in Table 1. The intervention groups were prescribed with 1%/food and 2%/food doses of NS and NCLN for 7 weeks, respectively. The powders were mixed with rat food and then pelleted. The exact amount of the prescribed supplements varied based on foods consumed.

2.7. Statistical analysis

Data is presented as mean (SD). The Kolmogorov-Smirnov test (KS) was applied for evaluating the normality of data distribution. For comparing study groups, if data was normally distributed, the mean values of OS and BW of rats were analyzed by ANOVA test. Otherwise, Kruskal-Wallis and appropriate post-hoc tests were applied. Data analysis was conducted using SPSS software version 16 and $p < 0.05$ was considered significant.

3. Results

3.1. Lipid peroxidation

Results revealed that MDA, SOD, GPX and TAC levels were significantly different ($p_{MDA} = 0.001$, $p_{SOD} = 0.002$, $p_{GPX} = 0.016$ and $p_{TAC} = 0.017$) between groups. MDA level, as one of the indicators of lipid peroxidation, increased significantly in the DC group in comparison to other groups (Table 3). According to results, treatment with NS and NCLN either separately or simultaneously prevented the increase of MDA level. Reduction in the amount of MDA was statistically significant in the NCLN ($p = 0.011$) and NCLN + NS ($p = 0.007$) groups but insignificant in the NS group ($p > 0.05$). In addition, concurrent use of NS and NCLN decreased MDA concentration to near control levels in diabetic NS + NCLN-treated rats. The higher increase in lipid

Table 3
Mean (SD) of antioxidant parameters in rats after interventions.

Groups	Treatment	MDA (nmol/mL)	SOD (u/mg Hb)	GPX (u/g Hb)	TAC (mmol/L)
1	DC (n = 6)	4.98 (1.52)*	2.42 (0.85)	2.68 (0.51)	1.44 (0.09)‡
2	NCLN + NS (n = 5)	2.82 (0.24)	0.75 (1.02)**	5.72 (3.19)	1.42 (0.13)
3	NS (n = 5)	3.70 (0.67)	3.24 (0.69)	7.06 (3.15)†	1.46 (0.11)‡
4	NCLN (n = 7)	3.08 0.59)	3.45 (0.56)	6.25 (1.89)†	1.38 (0.18)
5	NC (n = 6)	2.41 (1.04)	1.96 (1.66)	5.59 (1.95)	1.19 (0.13)

SD = Standard deviation, DC = Diabetic control, NCLN = Nano-sized clinoptilolite, NS = Nigella Sativa, NC = Normal control. ANOVA followed by Post-Hoc and Sidak tests.
* p value = 0.05 as compared to all other groups.
** p value = 0.05 as compared to NS and NCLN.
† p value = 0.05 as compared to DC.
‡ p value = 0.05 as compared to NC.

peroxidation in the diabetic group may indicate the longer period of exposure to the state of OS associated hyperglycemia.

3.2. Antioxidant enzyme activities and total antioxidant status

After two months of treatment, the activities of the antioxidant enzyme GPX in the NS and NCLN groups significantly increased by 2.63 and 2.33 fold compared to the DC and NC groups (7.06 ± 3.15 and 6.25 ± 1.89 *vs.* 5.59 ± 1.95 U/g Hb) ($p = 0.014$ and $p = 0.034$). This increase in the NS + NCLN group was insignificantly higher than the NC group (5.72 ± 2.19 *vs.* 5.59 ± 1.95 U/g Hb) ($p = 0.138$).

As shown in Table 3, the rate of TAC in the DC and NS groups, demonstrated significant increase as compared to the NC group ($p_{DC} = 0.031$ and $p_{NS} = 0.024$), but this increase was insignificant in the NCLN and NS + NCLN groups.

In all the diabetic subgroups except for NS + NCLN, SOD level was insignificantly higher than the NC group. Unexpectedly, SOD enzyme level was lower in the NS + NCLN group than the other groups and showed significant differences compared to the NS and NCLN groups ($p_{NS} = 0.007$ and $p_{NCLN} = 0.001$). It seems that simultaneous use of NS and NCLN had a negative impact on the level of this enzyme. According to results, the MDA/TAC ratio was significantly higher in the DC group (=3.46) than the NCLN + NS (=2), NS (=2.54), NCLN (=2.24) and NC (=2) groups.

3.3. Body weight (BW)

As shown in Fig. 2, there were no statistical significant differences in BW between groups before STZ injection and before the OGTT test. In the first week, only the differences between NC and NCLN ($p = 0.016$) were significant. In comparison to the NC group, a significant decrease ($p < 0.05$) in mean BW was observed for all the diabetic subgroups (excluding NS) at week 2, 3 and 6. Also BW decrease was observed in the DC and NCLN groups at the fourth and fifth week and for all the diabetic subgroups at the seventh week. However, no significant differences were observed between diabetic subgroups throughout the intervention process. Within-group comparisons showed a significant decrease in BW in the DC and NCLN + NS groups, ($p_{DC} = 0.029$ and pNCLN + NS = 0.011) at the seventh week compared to the fifth week. None of the treatments was able to significantly prevent weight loss caused by diabetes. Only a slight weight gain until the fifth week of treatment was observed in the rats treated with NS. There were no significant differences in proportion of mean food intake/body weight between groups during the study.

4. Discussion

Oxidative stress and consequent lipid peroxidation have harmful effects in relation to the pathogenesis of diabetes mellitus. Lipids and proteins are the primary target of OS [8,9]. Increased non-enzymatic glycosylation and auto-oxidation of glucose which induce lipid peroxidation and increase MDA concentration, are the possible mechanisms mentioned for the excessive production of free radicals in diabetes mellitus [26].

In the current study, induction of T2DM by HFD/STZ significantly increased MDA and TAC levels but decreased GPX. However; no significant changes were observed for erythrocyte SOD activities compared to the NC group. In our previous study, BG and MDA were significantly elevated in STZ-induced diabetic rats, while there were no statistical differences observed for SOD, GPX, and TAC [25]. Our results indicate significant reduction in BW in the DC rats, despite elevated food intake, during the experimental period. This finding may be because animals were undergoing growth retardation due to obstruction of glucose uptake followed by HFD/STZ intervention.

Regarding the effect of NS on lipid peroxidation and antioxidant defense system, the results of the present study showed a significant increase in Serum GPX and TAC levels in the NS group compared to the DC group. Furthermore, NS decreased MDA concentration favorably in HFD/STZ induced diabetic rats. Similarly, Kanter et al. reported that NS in STZ-induced diabetic rats, may protect pancreatic β-cells, and decrease MDA concentration in the pancreas and nitric oxide level in the Serum [27]. Through NS administration, tissue MDA level decreased and GSH level and SOD activity increased, indicating the anti-oxidative properties of the extract. These results suggest that NS reduces OS in kidneys. We can claim that NS prevented the depletion of antioxidant enzymes, including GSH. Kanter et al. revealed that treatment with volatile oil of NS decreased blood MDA levels and increased the anti-oxidative activity of the defense system in carbon tetrachloride-treated rats [28]. The antioxidant effect of NS may be attributed to its oil, TQ, flavonoids and also anti-oxidative vitamins such as ascorbic acid. It has been shown that NS oil and TQ scavenge various ROS, including superoxide anions and hydroxyl radicals. Moreover, flavonoids, a class of polyphenolic compounds, seem to have antioxidant properties by suppressing ROS formation and scavenging reactive oxygen and nitrogen species and also protecting the anti-oxidative defense system. Indeed, TQ successfully prevents the reduction of hepatic mRNA of PPAR-α and PPAR-γ induced by high fructose diet consumption. In addition TQ restores Ang II-inhibited expression of p-AMPK, PPARγ, and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) proteins and increases the expression of antioxidant genes, SOD, catalase and GPX. Other effects of NS such as scavenging superoxide, hydroxyl radical and singlet oxygenated molecules, induction of endogenous anti-oxidative enzymes by direct anti-oxidative effects, inhabitation of NF-κB and progressive apoptosis have been previously reported [29–32].

Our study demonstrated that dietary administration of NCLN, as a lipid per-oxidation (LPO) inhibitor and potential antioxidant, reduced MDA concentration and increased GPX, SOD and TAC contents. These results are in line with the results of Saribeyoglu et al., reporting that oral administration of CLN by gavage tube at doses of 5 mg/kg twice a day significantly reduced level of MDA in plasma and liver tissue, while SOD activity and GSH level increased in the liver tissue [10]. Positive effects of CLN treatment against HFD/STZ-induced OS were also observed in our study. Montinaro et al. showed that administration of clinoptilolite in water beverage (0.6, 1.25 and 2.5 ng/ml) for 5 months in an Alzheimer-induced mice model, significantly reduced the cellular death process caused by ROS in the group treated with clinoptilolite compared to the control group. A significant increase of ROS production in mitochondria, a significant rise in the activity of SOD enzyme in the hippocampus of mice, and a significant reduction in the level of amyloids plaques were also observed [33]. Although the exact anti-oxidative mechanisms of CLN are not well defined, the property of

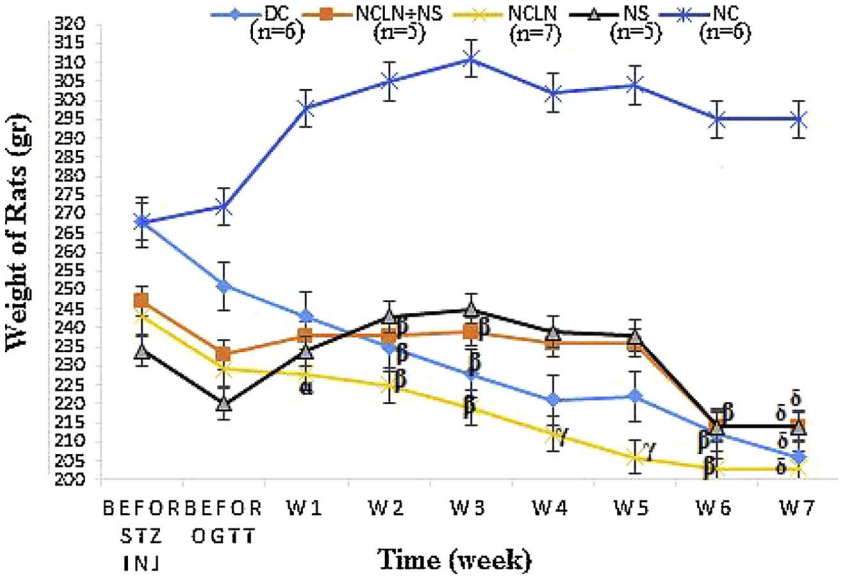

Fig. 2. Body Weight changes based on means (SD).
SD = Standard deviation, DC = Diabetic control, NCLN = Nano-sized clinoptilolite, NS = Nigella Sativa, NC = Normal control. α, β, γ and δ; p value < 0.05 as compared to NC group. Treatment by NS and/or NS + NCLN reduced severe weight loss to the fifth week of the intervention.

zeolite systems in neutralizing the solutions, acting either as proton acceptor or donor, have been recently described [32]. In the CLN-Fe oxidase system, CLN, as a zeolite, could intercept the production of peroxides and free radicals due to its amphoteric characteristic [10]. In addition, natural micronized CLN (TMAZ) can neutralize free radicals because it contains Cu, Mg or Zn, which are necessary for SOD production [34]. Altogether, NCLN could be considered as a novel class of LPO inhibitors or/and antioxidant.

In this study, the combination of NS and NCLN had different effects on OS and anti-oxidative status in comparison with separate treatments. The simultaneous use of these two materials significantly decreased MDA concentration than separate consumption of NS or NCLN compared to the diabetic control group. However, GPX increase was higher when NS or NCLN were used separately. Another finding of this study was the significant decrease of SOD enzymes in the NS + NCLN group compared to other groups. Decrease in SOD level by combination therapy was in contrast to our study hypothesis. This finding might be due to using hemoglobin for estimating SOD enzyme activity; it may be superior to perform other techniques to estimate the activity of SOD and also measure SOD activity in tissues.

According to results, diabetic rat's BW reduced during the 7 weeks experimental period, indicating that these animals were undergoing growth retardation due to obstruction of glucose uptake caused by lack of insulin. However, treatment with NS reduced BW loss until the last week of study. These results were in accordance with previous studies on NS [35].

In conclusion, our study provides novel evidence in support of separate and concurrent oral treatment of NS and NCLN as antioxidants in HFD/STZ-induced diabetic rats. Moreover, our results demonstrated that NS can reduce severe weight loss subsequent to diabetes. Future studies are needed to evaluate the effects of these two materials in T2DM with different dosages, longer duration and molecular and clinical settings. It is assumed that these materials, concurrently, could be effective on other diabetes-related disorders such as inflammatory and or immune system agents. Therefore, further well-designed studies are necessary to assess these parameters.

Conflict of interest statement

The authors declare no conflict of interest.

Statement of Human and Animal Rights

This article does not contain any studies with human subjects performed by any of the authors.

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