

Effects of zinc supplementation on superoxide dismutase activity and gene expression, and metabolic parameters in overweight type 2 diabetes patients: A randomized, double-blind, controlled trial

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ABSTRACT

Objective: Despite the current guidelines for the management of type 2 diabetes mellitus (T2DM), patients still struggle with the hyperglycemia consequences. Imbalance in zinc homeostasis, in particular, renders diabetic patients more susceptible to the damages of oxidative stress. This study aimed to evaluate the effects of zinc supplementation on the superoxide dismutase gene expression and enzyme activity in overweight individuals with T2DM. Additionally, biochemical parameters, such as fasting blood glucose (FBG), insulin, glycated hemoglobin (HbA1c), homeostasis model of assessment-insulin resistance (HOMA-IR), serum levels of zinc and lipid profile, were assessed.

Methods: In this randomized, double-blind, placebo-controlled trial, 70 overweight (BMI > 25) T2DM patients were selected based on the inclusion criteria. They were divided into two groups for supplementation of daily 50 mg zinc gluconate or placebo for 8 weeks. Blood samples were collected from all the individuals in the zinc group and controls for analysis.

Results: The results showed that, in comparison with the control group, zinc supplementation increased both gene expression and enzyme activity of SOD ($p < 0.01$) as well as the levels of insulin ($p = 0.02$) among the patients in the zinc group. Moreover, there was a meaningful reduction in the levels of FBG, HbA1c and HOMA-IR value ($p < 0.001$), triglycerides and total cholesterol ($p < 0.05$) after the zinc treatment.

Conclusions: Taken together, the current study suggests that daily supplementation with 50 mg zinc gluconate could be a useful approach for the management of overweight T2DM.

Clinical Trial Registration: IRCT2015083102.

1. Introduction

The prevalence of diabetes has been steadily growing worldwide and is expected to increase faster in developing countries. According to the WHO, the number of diabetic patients doubled from 1980 to 2014 [1]. The majority of these patients (about 90%) suffer from type 2 diabetes mellitus (T2DM) [2]. The risk of T2DM is mostly associated with a combination of lifestyle, old age, and metabolic factors [3]. Recently, there has been a growing interest in exploring the impact of various lifestyle aspects, including physical inactivity, overweight, and obesity in developing T2DM, with the findings indicating that being overweight or obese along with having a sedentary lifestyle are the primary risk factors for T2DM [4]. Obesity is seen in most T2DM patients, and to some degree, it can be a reason for the insulin resistance

[5]. In diabetic patients, chronic hyperglycemia causes glucose auto-oxidation and lipid peroxidation, leading to a rise of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Also, the observed changes in lipid profile in T2DM such as an increase in triglycerides and LDL-C as well as a reduction in HDL-C, can make patients susceptible to oxidative modification [6]. Accordingly, the ultimate result would be the imbalance of oxidant and antioxidant agents or oxidative stress, which plays a pivotal role in the pathogenesis of T2DM.

Zinc, as an essential micronutrient, plays an integral function in the metabolism of carbohydrates, lipids, and insulin [7]. It has a vital role in the proper function of many enzymes and transcription factors. There is substantial evidence that zinc metabolism alters in diabetic patients (impairs homeostasis). It has also been reported that zinc supplementation can ameliorate the condition of T2DM [8]. Both animal

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[9,10] and human studies demonstrated the beneficial effects of zinc on diabetes [11,12]. For example, Barman and Srinivasan showed that zinc supplementation alleviates the metabolic abnormalities resulting from hyperglycemia in diabetic rats [13]. This protective feature of zinc is mainly attributed to its anti-oxidant role and the improvement of insulin function [14]. Moreover, zinc enhances the antioxidant capacity, induces insulin sensitivity, and lowers serum glucose and lipids - known as the “insulinomimetic effect” [15]. Superoxide dismutase, in particular, is a zinc dependent enzyme and has an active center with a zinc ion and a copper ion, (CuZn-SOD) or (SOD1); that is, zinc forms part of the structural integrity and maintenance of the SOD molecule [16]. As a part of the antioxidant defense system, SOD catalyzes superoxide (O_2^-) to H_2O_2 , which is subsequently transformed into harmless products [17]. Hence, impaired zinc homeostasis adversely affects the synthesis and function of the antioxidant defense system, leading to oxidative stress. Evidence indicates that dyshomeostasis and increased urinary loss of zinc are found in diabetic patients [14,18]. Notably, chronic hyperglycemia causes glycation in the SOD enzyme, leading to impaired enzyme activity [19]. As a consequence, extremely active radicals (such as hydroxyl, reactive ketoaldehydes, and superoxide anions) undergo dismutation, resulting in irreversible cell damage in long-term hyperglycemic conditions [20]. Due to the role of oxidative stress damage in the pathogenesis of T2DM, zinc supplementation (as a candidate for anti-oxidant therapy) has drawn researchers' attention. Recently, there have been many studies investigating the effect of zinc supplementation in diabetes, yet the outcomes are controversial. Moreover, it is still not clear whether zinc affects both SOD enzyme activity (short-term effect) and mRNA expression (long-term effect). Some studies have suggested that the beneficial effects of zinc are independent of SOD enzyme activity [21], while others have concluded that zinc may have a short-term influence on SOD activity [22].

In this study, our objective was to assess the effect of zinc supplementation on the management of overweight T2DM patients, the SOD enzyme activity and gene expression of SOD, as well as on various markers of glycemic control and lipid profiles.

2. Materials and methods

2.1. Participants

We carried out a matched, randomized, double-blind, placebo-controlled clinical trial from January to June 2018, with eighty overweight participants with a confirmed diagnosis of T2DM of at least five years. These patients had been referred to the Diabetes and Metabolic Diseases Clinic of Endocrinology and Metabolism Research Institute, Tehran, Iran. All participants had inclusion criteria that included obesity ($BMI = 25\text{--}30\text{ kg/m}^2$) male or female, 40 to 65 years old, and a diagnosis of T2DM based on American Diabetes Association criteria ($FBG \geq 126\text{ mg/dl}$ and $HbA1c \geq 7\%$) [1]. The exclusion criteria included cigarette smoking, any history of cancer, thyroid or gastrointestinal disorders, a record of taking diuretics, antibiotics, insulin therapy or taking vitamin/mineral supplements containing zinc less than two months before the beginning of the study. Ten patients did not agree to participate in this trial, hence we proceeded with seventy participants. This trial was registered at the Iranian Registry of Clinical Trials (IRCT registration number: IRCT20150831023838N1), and all procedures were approved by the Research Ethics Committee of Endocrinology Metabolism Research Institute, Tehran University of Medical Sciences (IR.TUMS.EMRI.REC.1395.00105).

2.2. Zinc supplementation and sample collection

Seventy patients were randomly allocated to two groups using permuted block randomization: treatment (zinc) group ($n = 35$), or control (placebo) group ($n = 35$). The treatment group received a 25 mg zinc capsule (in the form of zinc gluconate, Alhavi Pharma, Iran)

twice daily and the control group received a placebo twice daily (Avicel capsule, Sigma). Both groups took the capsules after their main meals, under the supervision and prescription of an endocrinologist for eight weeks. In addition, all the participants received the same dietary plan, as prescribed by a professional dietitian. All the patients were contacted once a week during this study to ascertain compliance with the program. All the patients were visited twice during the study by the endocrinologist and there was no noticeable report of any side effects from the zinc treatments, such as diarrhea, headache, vomiting, nausea or copper deficiency. Before and after the eight weeks of supplementation, fasting blood samples were taken from each participant (7 mL). Blood specimens were collected into EDTA tubes (2 ml) and tubes without additive (5 ml) for the gene analysis and biochemical assays, respectively. The samples were centrifuged immediately after collection at 3500 rpm for 10 min, the serum and plasma were divided into small aliquates and kept at $-80\text{ }^\circ\text{C}$ until analysis.

2.3. Laboratory parameters

The serum concentrations of FBG and triglyceride, total cholesterol, HDL-C, LDL-C were determined using commercially available kits (Pars Azmoon, Tehran, Iran) on the Biolis 24i Premium (DiaSystem, Husqvarna, Sweden). Serum levels of insulin were assessed using Electrochemiluminescence (ECL) method (Elecsys 2010 Immunoassay Analyzer, Roche, Switzerland). Serum zinc levels were determined by atomic absorption spectroscopy (Shimadzu AA-670, Tokyo, Japan with analytical range: $1\text{--}150\text{ }\mu\text{g/dL}$) and HbA1c was measured with G8 HPLC Analyzer (Tosoh Bioscience, San Francisco, USA). Plasma SOD enzyme activity was assessed using the SOD assay kit (ZellBio, GmbH, Germany). Insulin resistance was calculated according to the Homeostatic model assessment of insulin resistance (HOMA-IR) method: $\text{fasting insulin } (\mu\text{U/mL}) \times \text{fasting blood glucose } (\text{mg/dL}) / 405$ [23].

2.4. RNA isolation, real-time PCR, and SOD gene expression analysis

Leukocyte RNA was extracted and purified using an RNA isolation kit (Nucleospin RNA Blood, Macherey-Nagel, Duren, Germany), according to the manufacturer's instructions. RNA concentration was assessed by absorbance at 260 nm, and its integrity determined by agarose gel electrophoresis. Then, RNA was reverse-transcribed using a cDNA synthesis kit (PrimeScript RT reagent kit, TAKARA BIO INC, Kusatsu, Japan), and cDNA samples were stored at $-20\text{ }^\circ\text{C}$ until required for further analysis. Specific primers for SOD1 (CuZn-SOD) and β -actin (housekeeping gene) were designed by Primer Blast (NCBI), evaluated by Oligo Primer Analysis Software v.7 (Molecular Biology Insights, Inc., USA), and synthesized by Macrogen Co. (Seoul, Korea).

The primers sequences were as follows: forward; $5'\text{-GGGCAATGT GACTGCTGACAAAGATGG-3'}$; reverse, $5'\text{-CTTCTTCATTCCACCTT GCCCAAGTC-3'}$ for SOD1 and forward; $5'\text{-GCAAGCAGGAGTATGACG CTAG-3'}$; reverse, $5'\text{-GTCACCTTCACGGTCCAGTGTC-3'}$ for β -actin as a housekeeping gene. Real-time PCR was performed in the Exicycler 96 (Bioneer, Daejeon, Korea) with a reaction mixture that consisted of SYBR Green $2 \times$ PCR Master Mix (RealQ Plus Master Mix Green, Ampliqon, Stenhusgervej, Denmark), cDNA template, forward primer and reverse primer. Then, the quantitative PCR (qPCR) was carried out with the following protocol: initial denaturation at $95\text{ }^\circ\text{C}$ for 5 min, followed by 35 cycles (denaturation at $95\text{ }^\circ\text{C}$ for 30 s, annealing $62\text{ }^\circ\text{C}$ for 30 s and extension $72\text{ }^\circ\text{C}$ for 1 min). Finally, the amounts of SOD mRNA were normalized to β -actin, and fold change in relative gene expression was calculated using the Livak formula, $2^{-\Delta\Delta Ct}$ [24].

2.5. Statistical analysis

SPSS 16 (IBM Corp.) was utilized for data analysis. Descriptive data were expressed as the mean \pm standard deviation (SD). The normality

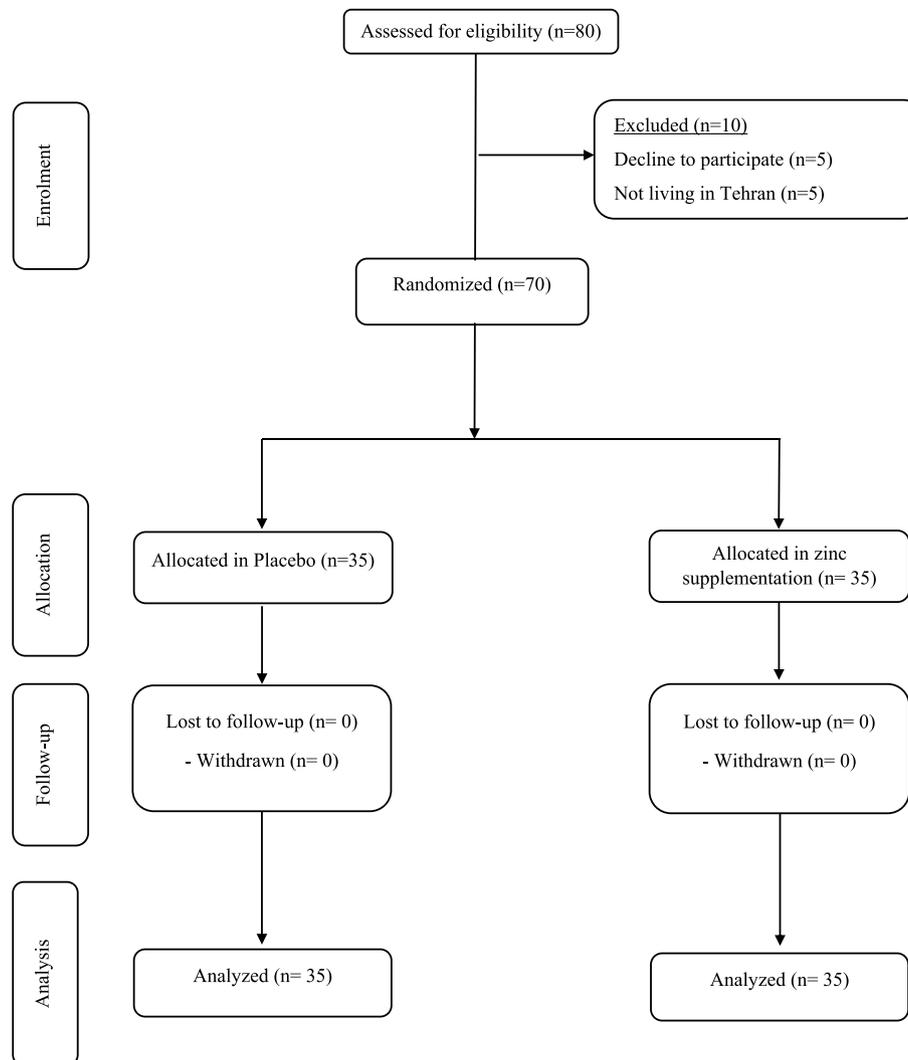


Fig. 1. The summary of participants' flow chart.

of variable distribution was checked by the Kolmogorov-Smirnov test. Independent *t*-test or Mann-Whitney test were used to compare the mean of continuous variables between zinc and placebo groups. Furthermore, analysis of covariance (ANCOVA) was carried out to compare post-treatment variables after adjusting the baseline values in both groups. Correlations between variables were assessed by Pearson's correlation analysis. A *P*-value of < 0.05 ($p < 0.05$) was considered as statistically significant.

3. Results

3.1. Baseline characteristics of participants

Seventy participants with the mean age of 55.42 ± 8.71 years old followed and completed the study. The zinc group consisted of 35 participants (16 female and 19 male), and there were 35 subjects in the placebo group (16 female and 19 male). Fig. 1 demonstrates the flow of study. Two study groups were matched regarding Metformin and Glucophage usage as well as the duration of T2DM (Table 1). Comparing the two groups, zinc supplementation significantly decreased the mean weight and subsequently the mean BMI (Table 2).

3.2. Biochemical parameters

In the zinc group, after zinc supplementation, there was a

statistically significant increase in the serum levels of zinc, while there was not a significant alteration of this parameter in the control group (Fig. 2). As illustrated in Table 3, the zinc group demonstrated lower levels of FBG, HbA1c, triglyceride, total cholesterol and LDL-C, whereas the levels of HDL-C and insulin were higher after zinc supplementation.

3.3. SOD mRNA expression and enzyme activity

Data from qPCR revealed that two-month daily 50 mg zinc gluconate intake up-regulated the expression of SOD 2.33-fold ($p < 0.001$) compared to the placebo group (Fig. 3a). The activity of the enzyme was also assessed with the plasma SOD assay kit. Likewise, as demonstrated in Fig. 3b, SOD activity was significantly increased due to zinc treatment ($p < 0.01$). Conversely, in the case of the placebo group, the SOD activity did not change during the study period.

3.4. Correlation studies

As shown in Fig. 4a, the Pearson's correlation analysis showed that the serum zinc levels were correlated with the SOD enzyme activity. Likewise, there was a correlation between the serum zinc concentrations and the SOD expression at mRNA levels (Fig. 4b).

Table 1
Baseline characteristics of the participants in the two groups.

	Age (year)	Duration of T2DM (year)	Metformin (mg/day)	Gliclazide (mg/day)
Zinc group	53.28 ± 7.35	10.12 ± 3.46	1071.43 ± 366.67	34.86 ± 21.74
Placebo group	54.34 ± 7.18	9.77 ± 3.15	1057.14 ± 338.06	34.57 ± 21.05
p-value	<i>p</i> = 0.99	<i>p</i> = 0.13	<i>p</i> = 0.86	<i>p</i> = 0.95

Independent t-test was used. Values are expressed as mean ± S.D.

Table 2
Weight and body mass index, before and after zinc or placebo supplementation.

	Zinc group		Placebo group		*p-Value
	Before	After	Before	After	
Weight (kg)	78.20 ± 12.30	75.49 ± 11.54	79.40 ± 9.66	80.20 ± 9.82	<i>p</i> < 0.001
BMI (kg/m ²)	27.61 ± 1.94	26.72 ± 2.04	28.18 ± 1.90	28.50 ± 2.01	<i>p</i> < 0.001

BMI: body mass index.

* P-value of variables after receiving zinc supplement vs. placebo obtained through conducting ANCOVA. Values are expressed as mean ± S.D.

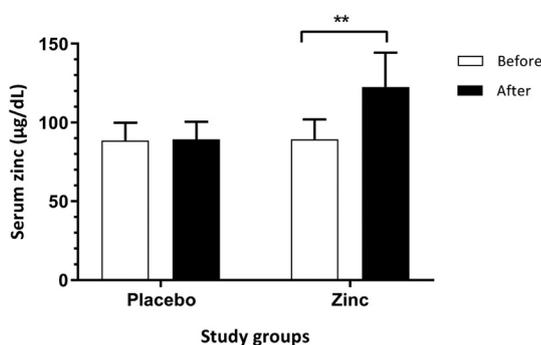


Fig. 2. Serum zinc concentrations before and after zinc or placebo supplementation in study groups.

P-value of the variable after receiving zinc supplement vs. placebo obtained through conducting ANCOVA.

** Significant (*p* < 0.001).

4. Discussion

In this randomized, matched, double-blind, placebo-controlled trial, the effects of eight-week daily 50 mg zinc gluconate supplementation on overweight individuals with T2DM has been examined. There is compelling evidence that zinc homeostasis is disrupted in T2DM [25,26]. Even though the recommended daily allowance (RDA) for zinc in both men and women is 11 mg/day [15], many preclinical and clinical studies suggest that higher zinc levels through oral

supplementation can be more effective, particularly in diabetic patients [27–30]. Accordingly, 50 mg zinc gluconate (25 mg twice daily) was prescribed to be taken after the main meals. The participants in this study showed serum zinc levels between 70 and 120 µg/dL which is considered as the normal range at the baseline. However, zinc supplementation resulted in a significant increase (37%) in the serum level of zinc.

One possible approach to evaluate the positive impact of zinc intake in T2DM patients is to monitor glycemic control markers, such as FBG, HbA1c, insulin, and HOMA-IR [8]. Our findings showed that patients who received zinc supplements for eight weeks had noticeable decreases in FBG, HbA1c, HOMA-IR and a significant rise in serum insulin concentrations compared with the placebo group. In other words, all glycemic markers effectively improved. On the other hand, there are controversial reports about the effect of zinc on glycemic control markers among T2DM patients [21]. Despite the elevated levels of serum zinc, some studies failed to demonstrate a positive effect on glycemic markers [31,32]. Participants' features (like age and health conditions), supplementation period, variation in the chemical form of zinc (zinc sulfate or zinc gluconate) as well as the difference in doses may account for the discrepancies among reports [18].

In parallel, we observed a positive influence of zinc intake on lipid profiles. Zinc supplementation lowered serum levels of total cholesterol, LDL-C and increased serum levels of HDL-C in the zinc group. These findings are consistent with the results of previous studies showing the positive effect of zinc on lipid profiles in T2DM patients [33]. For example, a meta-analysis study by Ranasinghe, P. et al. reported that most clinical trials showed that zinc has a beneficial effect

Table 3
Changes in biochemical parameters before and after zinc or placebo supplementation.

	Zinc group		Placebo group		*p-Value
	Before	After	Before	After	
FBG (mg/dL)	161.23 ± 31.43	121.94 ± 21.15	148.31 ± 24.25	149.43 ± 25.84	<i>p</i> < 0.001
HbA1c (%)	7.70 ± 0.63	7.35 ± 0.57	7.51 ± 0.58	7.56 ± 0.55	<i>p</i> < 0.001
Insulin (µU/mL)	13.41 ± 4.95	14.45 ± 4.85	13.61 ± 5.33	13.50 ± 5.09	<i>P</i> = 0.02
HOMA-IR	5.25 ± 1.92	4.35 ± 1.67	5.03 ± 2.41	5.01 ± 2.24	<i>p</i> < 0.001
Triglycerides (mg/dL)	161.89 ± 48.96	155.51 ± 44.56	174.46 ± 46.74	191.81 ± 54.42	<i>p</i> = 0.01
Total cholesterol (mg/dL)	161.54 ± 32.67	149.69 ± 27.22	156.00 ± 30.36	152.89 ± 28.88	<i>p</i> = 0.04
LDL-C (mg/dL)	81.91 ± 22.51	76.26 ± 18.39	71.51 ± 17.19	76.63 ± 12.55	<i>p</i> = 0.01
HDL-C (mg/dL)	41.34 ± 8.13	45.94 ± 7.49	39.83 ± 9.20	41.03 ± 8.34	<i>p</i> < 0.001

FBG: fasting blood glucose, HbA1c: Hemoglobin A1c, HOMA-IR: homeostasis model of assessment-insulin resistance, HDL-C: high-density lipoprotein-cholesterol, LDL-C: low-density lipoprotein-cholesterol.

* P-value of variables after receiving zinc supplement vs. placebo obtained through ANCOVA except for FBG and HOMA-IR with non-normal distribution, analysed by Mann-Whitney. Values are expressed as mean ± S.D.

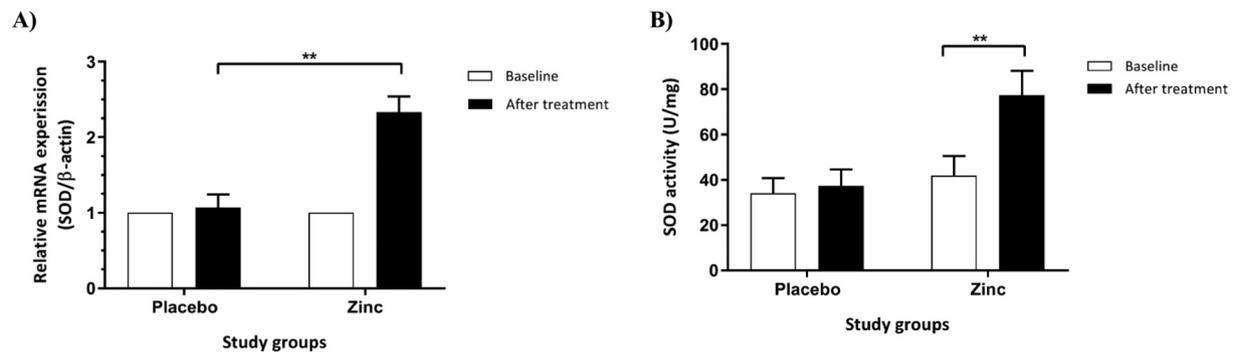


Fig. 3. A) SOD gene expression. The expression of SOD gene was normalized to β -actin and presented as arbitrary units compared with the placebo group (***) $p < 0.001$). B) SOD enzyme activity. Enzyme activity increased after zinc supplementation (** $p < 0.01$). Data are the mean \pm S.D. of duplicates from an experiment that was repeated with similar results.

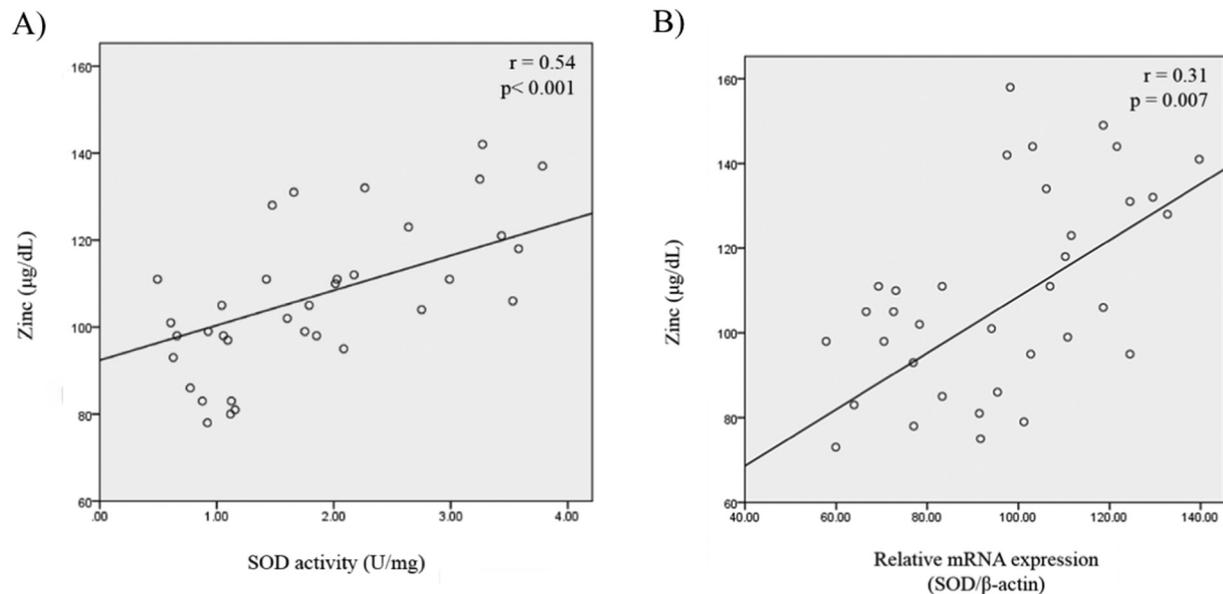


Fig. 4. correlation studies. A) The correlation between serum zinc and SOD enzyme activity in the zinc group after treatment. B) The correlation between serum zinc and SOD gene expression in the zinc group after treatment.

on lipid markers [34,35].

Obesity management is one of the primary concerns in T2DM therapy [5]. To examine how zinc supplementation can be beneficial for weight control all the participants were chosen from the overweight population. Considering weight loss following the treatment in the zinc group (Table 2), this supplementation may affect weight and BMI. The data may suggest that lipid profiles and glycemic markers are directly affected by zinc supplementation or possibly are affected as a consequence of the weight loss.

Different mechanisms have been suggested for the beneficial effects of zinc on T2DM: 1- The enhancement of insulin function, 2- The improvement of antioxidant capacity, 3- Inhibition of lipid peroxidation [35,36]. For example, Jansen, J. et al. pointed out that zinc functions by enhancing insulin signaling [37]. More specifically, high levels of zinc improve SOD activity, ultimately leading to the balance of anti-oxidant agents [38,39]. Nevertheless, it should be taken into account that most of these research studies have been carried out using animal models or cell lines, and a limited number of studies have scrutinized the molecular mechanisms of zinc in the diabetes management in humans [40]. In other words, zinc is considered to favorably influence oxidative stress and lipid peroxidation, but there was a paucity of information whether zinc could positively affect both gene expression and enzyme activity of SOD [41]. Here, we showed that the degree of SOD gene expression along with SOD enzyme activity increased in T2DM patients following

8 weeks of supplementation with oral zinc. The correlation of serum zinc levels with SOD gene expression and SOD enzyme activity suggests that through the up regulation of SOD gene, zinc might be able to directly increase the activity of SOD enzyme, improve its function, and ultimately enhance the antioxidant defense system.

5. Limitations

There are some limitations to the current study. Due to ethical considerations in clinical interventions, our sample size was quite small. Based on the inclusion and exclusion criteria, we were unable to assess a broader population of diabetic patients. Moreover, although correlations between zinc levels, SOD gene expression, and SOD enzyme activity were observed, future research with a larger sample size is required to verify this relationship. Also, additional studies may help to understand if there is a direct effect of zinc supplementation on other factors such as weight loss.

6. Conclusion

To the best of our knowledge, this study is the first indicating zinc has a significant effect on SOD activity and SOD mRNA expression. Overall, regarding the significance of adjunct therapy besides applying primary medications such as insulin and metformin, it was

demonstrated that zinc supplementation could be a safe and efficient treatment to control lipid and glycemic markers in T2DM patients, and it can improve antioxidant status due to its effect on SOD activity.

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Declarations of interest

None.

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