Effects of zinc, vitamin D, and their co-supplementation on mood, serum cortisol, and brain-derived neurotrophic factor in patients with obesity and mild to moderate depressive symptoms: A phase II, 12-wk, 2 × 2 factorial design, double-blind, randomized, placebo-controlled trial

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Keywords: depression, obesity, BDNF, cortisol

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1. Introduction:

Mood disorders, particularly depression, are one of the most prevalent mental health symptoms in patients with obesity (1-3). Several studies have reported that patients with obesity can suffer with major depression (4-6). Although the direction of the association between depression and obesity has been questioned (3, 7, 8), the co-occurrence of both may have a detrimental synergistic effect on overall health and treatment response (9). A growing evidence base indicates that there are abnormalities in the hypothalamic-pituitary-adrenal axis, particularly in the regulation of cortisol, in clinically depressed patients, which is, in turn, related to obesity (10-12). Moreover, empirical evidence has suggested that Brain-derived neurotrophic factor (BDNF), a homo-dimer protein, has a critical role in the pathophysiology of depression (13).

Despite representing the most preponderant treatment for depression, antidepressant medications have been reported to show resistance in depression associated with obesity (14, 15), whilst remission is only achieved in one-third of the patients after treatment with antidepressant agents (16). Furthermore, pharmacotherapy is usually costly (16-18). The expense and incongruent effectiveness in patients with obesity highlights the need to investigate alternative preventive and treatment approaches to traditional antidepressant medication. In recent years, it has been shown that nutritional intervention can be considered as an effective alternative or adjunct, preventive or treatment strategy to pharmacotherapy in depression (19). In particular, Zinc and vitamin D have been well linked with the treatment or management of depression (20-23).

Zinc deficiency can induce depressive-like behavior, and in this instance, the symptom can be effectively reversed by zinc supplementation (24, 25). Zinc may produce antidepressant-like effects by modulating the functions of the hypothalamus–pituitary–adrenal (HPA) axis and increasing serum BDNF (13, 26-28). Moreover, there is wealth of literature suggesting zinc supplementation is an effective adjunct therapy for major depressive disorders (13, 29, 30). Similar to zinc, inadequate vitamin D intake has been associated with depression (31, 32); whilst it has also been demonstrated that multiple brain regions are associated with depressive disorders, including the prefrontal cortex and hippocampus, and possess specific nuclear receptors for 1,25(OH)D (32, 33).

Although there have been significant advances in understanding the potential role of zinc and vitamin D in depression (13, 23), the literature, particularly in regard to vitamin D, is equivocal (34-36). Whilst, to date, only one randomized control trial has examined the effects of zinc

monotherapy on BDI-II score in obese patients, which resulted in a decreased BDI-II score (13). In addition, only one study has examined the effects of vitamin D monotherapy on BDI-II in obese patients with pre-existing depressive symptoms, where the authors suggested that supplementation of high doses of vitamin D can ameliorate depression symptoms (23). However, the results other studies investigating the effect of vitamin D supplementation on depression are divergent (37-39), and may be mediated by the inconsistent doses and duration of vitamin D supplementation.

Several meta-analyses have evaluated effect of zinc/vitamin D on depression (40-43) , concluding that, although there is evidence supporting their use, available research needs to be confirmed by larger RCT and prospective cohort studies (44-46). Furthermore, the effects of vitamin D or zinc on depression have mostly been explored independently in previous studies (13, 23), and to the author's knowledge, no study has examined the effects of zinc- vitamin D co-supplementation on depression symptoms in obese subjects. Interestingly, there is evidence to suggest that zinc homeostasis and function may be increased following vitamin D supplementation, and that the control of zinc in systemic levels is regulated by vitamin D (47). The current trial hypothesized that there may be additive benefits from combining zinc and vitamin D. Thus, the present study sought to investigate the effects of zinc, vitamin D, and their combination, on depression score, serum BDNF, and cortisol level in obese patients with mild to moderate depression.

2. Material& method

2.1. participants

The present study was a 12-week 2 × 2 factorial design randomized double-blind placebo controlled trial, and was conducted among 140 overweight/obese (BMI> 27 kg/m^2) adult subjects aged > 20 years with BDI-II≥10. Obese/overweight subjects were recruited from patients who were referred to the Endocrinology and Metabolism Research Center (EMRC), Vali-Asr, Emam khomeini Hospital in Tehran, Iran between July 2016 and February 2017. The depression status was evaluated by Beck Depression Inventory-II (BDI-II) questionnaire, and those who had a BDI-II score greater than 10 points were considered as eligible for current the trial. We excluded patients who had a history of psychiatric and neurological disorders (such as schizophrenia Parkinson's, Alzheimer's disease, anxiety, suicidal thoughts), coronary artery disease, acute or chronic renal failure, acute or chronic hepatic failure, chronic inflammatory and autoimmune disease, or any known malignancy, had been received antidepressant

medicines (in the preceding 3 months), or steroid or hormone-therapy. Other non-pathological exclusion criteria included pregnancy, breastfeeding, post-menopause, smoking, professional athlete, uncontrolled thyroid disorder, >3 kg weight change during the last 3 months, use of medications for dyslipidemia or hypertension, hypnotics, sedatives and immunosuppressive, any kind of supplements or following a special diet prescribed by the clinic dietitian. It was explained to each participant that the dose and type of supplement(s) assigned must not be changed during the intervention period, or they would be excluded from the study.

2.2. Ethics statements

This trial was performed according to the principles of the Declaration of Helsinki, and the study protocol was approved by the institutional ethics committee of Iran University of Medical Sciences (IR.IUMS.REC 1395.9221324205). The study protocol was carefully explained to all subjects signing an informed consent form. This trial was registered in the Iranian Web site (www.irct.ir) for registration of clinical trials (http://www.irct.ir: IRCT201601252394N31- 31-10-2016). The project was financially supported by Iran University of Medical Sciences.

2.3. Study design and intervention

Randomized assignment was performed using balanced the block randomization method. Block randomization works by randomizing participants within blocks such that an equal number are assigned to each treatment arm. Block randomization is a commonly used technique in trials with small sample sizes (48). In the present trial, the block size was 8. Participants were initially randomized to 'intervention vs Placebo,' and then assigned to one of the four groups via the balanced block randomization method (in a 1:1:1:1 ratio), (1) subjects received 2000 IU vitamin D3 daily plus a daily placebo for zinc; 2) subjects received 30 mg zinc gluconate per day plus a daily placebo for vitamin D; 3) subjects received 2000 IU vitamin D3 daily plus 30 mg zinc gluconate per day; or 4) subjects received identical matching placebos for vitamin D and zinc for 12 weeks. At the beginning of the study, participants were requested not to change their routine lifestyle throughout the study and not to consume any supplements or medication other than that provided to them by the investigators. The doses of vitamin D3 and zinc were chosen after a comprehensive review of the available literature to achieve optimal efficacy and safety (13, 49). The appearance of the placebo was indistinguishable in color, shape, size, packaging, and taste from vitamin D and zinc tablets. The zinc supplements were tablets manufactured by the Jalinus Pharmaceutical Company (Tabriz, Iran) and the placebo (made from starch) was provided by the School of Pharmacy, Tehran University of Medical Sciences. The vitamin D supplements and placebos (made from starch) were tablets

manufactured by Pars mino Company (Tehran, Iran). All research staff including investigators and laboratory technicians, as well as participants, were blinded to the random allocation.

2.4. Treatment adherence

The tablets were supplied to the participants, fortnightly, by the trial staff. Each bottle contained 15 tablets, and a tablet count was performed fortnightly by the investigator to assess compliance. To increase the adherence, all participants received short messages on their cell phones to take the supplements every day.

2.5. Assessment of variables

All assessments and measurements were made at study baseline and after the intervention period. Weight (in kilograms −kg) was measured using a calibrated Seca scale (Model 700, USA) with participants in light clothing and unshod. Standing height was measured to nearest 1 cm using a Seca stadiometer (Model 700, USA) while subjects were unshod. Body mass index (BMI) of each participant was calculated as body weight divided by height squared (kg/m^2). Waist circumference was measured using a flexible tape at the smallest circumference around coastal margin. A professional nurse measured the systolic and diastolic blood pressure on the non-dominant brachial artery, with the participants in a seated position, after having rested at least for 10 minutes. Blood pressure was measured twice, and the average of 2 measurements was considered as the final systolic and diastolic blood pressure. A trained researcher completed questionnaires on participants socio-demographic background and physical activity, whilst a researcher provided a comprehensive explanation of how to complete the self-rating BDI-II questionnaire. The trial used the validated Persian version of Beck depression inventory-II (BDI-II) (50)

To assess serum 25(OH) D, zinc, BDNF and cortisol levels, blood samples (10 cc) were obtained after a 12-hour overnight fast at study baseline (week 0) and after intervention (week 12). All blood samples were centrifuged at 3000g for 10 minutes, the serum was separated into clean tube aliquots, and were stored at $-80^{\circ C}$ until analysis at the Iran University of Medical Sciences Reference Laboratory. Serum 25(OH)D concentrations were quantified using the enzyme-linked immunosorbent assay (ELISA) method (Euroimmun,). The inter- and intra-assay coefficients of variation of this method were 8.6% and 3.2%. respectively. A serum 25(OH) D level <75 nmol/l (<30 ng/ml) was considered insufficient. ELISA methods were used to measure serum cortisol (diametra, Italy). The inter- and intra-assay coefficients of

variation of this method were 11% and 5.1%. respectively. Serum BDNF was quantified using the ELISA method (crystalday, china). The inter- and intra-assay coefficients of variation of this method were 10% and 8% respectively.

2.6. Sample size

To calculate sample size, we used the standard formula suggested for factorial design, clinical trials by considering type I error (a) of 0.05 and type II error (b) of 0.20. Based on a previous study (13), we used 5.698 as the standard deviation and 2.92 as the mean difference in BDI-II score as a variable primary outcome. Based on this, we required 33 persons in each group. Accounting for 2 dropouts (effect size: 0.52) in each group, the final sample size was determined to be 35 persons per group.

2.7. Statistical methods

We evaluated changes in depression symptoms as a primary outcome, during an average of 12 weeks of follow-up, in those randomized to zinc-vitamin D and their combined supplementation compared to placebo. The Kolmogorov–Smirnov test was used to examine and confirm the normal distribution of variables. The analyses were performed based on a per-protocol approach. One-way analysis of variance (ANOVA) was used to detect differences in general characteristics, blood pressure and anthropometric measurement, at the study baseline and post-intervention between the groups. To estimate the effect of zinc, vitamin D and their combination on outcomes, we first computed the changes from baseline by subtracting the baseline value from the end-of-trial value, and then applied analysis of co-variance (ANCOVA; adjusted by baseline serum zinc, vitamin D, cortisol, BDNF and beck depression score). To ensure that baseline characteristics balance was achieved by the randomization, analyses was conducted for baseline characteristics (zinc, vitamin D, age, sex, BMI, weight, height). In cases of minor imbalances, adjustment for these variables will be made our analyses. For all tests, statistical significance was accepted at $p \leq 0.05$. All analyses were performed using the Statistical Package for Social Sciences version 21 (SPSS, Chicago, IL, USA).

3.Results:

The present study was conducted using 140 obese patients with mild to moderate depression. In total, 15 patients declined to complete the intervention, and were therefore excluded from

subsequent analysis (**diagram 1**). Dropout rate was not significant between the treatment arms (P= 0.785). Serum levels and tablet counts of zinc and vitamin D at the end of the intervention suggested that the compliance was excellent. No significant differences in demographic characteristics were observed among the 4 intervention arms at baseline. Moreover, there was no significant difference in anthropometric measurements, such as weight, BMI, waist circumference (WC) and blood pressure, in zinc, vitamin D and co-supplementation groups compared to placebo ($p>0.05$). We found a significant decrease in blood pressure among those who received vitamin D (systolic blood pressure: 121.61±13.81 vs 118.00±15.76, P=0.0001. diastolic blood pressure: 81.96±10.00 vs 84.58±9.79, P=0.014) or joint zinc–vitamin D (systolic blood pressure: 119.26±14.07 vs 114.73±14.66, P=0.0001. diastolic blood pressure: 80.30±10.80 vs 78.73±11.94, P=0.0001) supplements. A significant decrease in WC was shown in vitamin D (106.91±11.39 vs 105.33±11.69, P=0.0001), zinc (103.63±5.84 vs 103.14±6.38, P=0.0001) and combined zinc–vitamin D (105.25±8.95 vs 103.85±9.20, P=0.0001) groups. The baseline and post-intervention characteristics of the study population are presented in Table 1. There was no evidence that supplementation elicited any injurious or negative side effect(s).

The effects of vitamin D, zinc and combined zinc–vitamin D supplementation on BDI-II, serum cortisol and BDNF are detailed in Table 2. No significant differences in BDI-II score were observed among the 4 intervention arms at baseline. The baseline mean of the BDI-II score was 19.21±7.34, for the whole population. All subjects had BDI-II≥10 at baseline, and after 12 weeks intervention, 45.2%, 66.7%, 59.4%, and 86.2% of participants in zinc, vitamin D, zinc-vitamin D and placebo groups, respectively. had BDI-II≥10. Zinc, vitamin D, and their co-supplementation yielded a significant reduction in depression score ($p<0.0001$). However, a greater reduction in depression score was observed in the zinc group compared with vitamin D group ($p<0.001$). No significant changes in BDI-II score were observed in placebo group ($p=0.396$).

We found significant differences in serum zinc ($p<0.001$), vitamin D (table 3) ($p<0.001$), cortisol ($p=0.049$) and BDNF ($p=0.004$) (table 2) between the study groups at baseline. The baseline mean of BDNF, cortisol, zinc, and 25-(OH) D levels were 2.54±1.61ng/ml, 16.52±5.87 ng/ml, 74.85±36.36mg/dl, and 18.99±12.02ng/ml respectively, for the whole sample population. We found a significant increase in serum zinc, and 25-(OH) D levels among those who received zinc, vitamin D or joint zinc–vitamin D supplements. However serum 25-(OH) D levels was also significantly reduced in the placebo group ($p< 0.001$).

Zinc, vitamin D or zinc+vitamin D had no significant effects on serum cortisol level. There was a significant decrease in serum BDNF levels in the zinc (p=0.035) and placebo (p=0.016) groups, respectively; whilst there was no significant change in the vitamin D group.

4.Discussion:

In the present study, we found that 3 months of supplementation with 30 mg/day of zinc gluconate, and 2000 IU vitamin D, either individually or in combination, significantly improved depression status compared to placebo in obese subjects who had BDI-II≥10. However, among these participants we found that, monotherapy with 30 mg zinc gluconate outperformed 2000 IU vitamin D in improving the depression-related symptoms over 12 weeks of follow-up. There was no significant differences in BDI-II score among the 4 intervention arms at baseline. Our results suggest that depressed patients could take zinc, vitamin D, either individually or concurrently, to improve mood and depression severity. Previous studies have shown that vitamin D or zinc supplementation, individually, may improve depression severity (13, 23), although this has been disputed in some empirical work (34-36).

Although favorable effects from zinc supplementation on depression-related symptoms have been demonstrated in previous clinical trials (13, 51, 52), some work has reported that there is no evidence for the significant association between zinc and depression (34). Importantly, it should be noted that the aforementioned clinical trials were generally conducted in people who were not affected by obesity. Thus, the role of supplemental zinc in obese patients with depression remains controversial. One meta-analysis showed that zinc concentrations were approximately −1.85mmol/L lower in depressed subjects vs. control subjects (46). Similarly, Zongyao Li noted a significant inverse association between dietary zinc intake and risk of depression (44). The current trial suggests a causal association between zinc status and depression in obese/overweight subjects, and that greater depression improvement is manifest in zinc, compared to vitamin D, supplementation.

In the present study, we found that vitamin D elicited a favorable effect on depression scores in overweight/obese subjects. Findings in some earlier clinical trials regarding the effect of vitamin D supplementation on depression have been inconsistent (23, 35, 53). A large randomized trial in older women assessed the effect of a single annual dose of 500 kIU vitamin D for 3–5 y, and did not find any effect of vitamin D supplementation on depression symptom (35); whilst further work reported that weekly supplementation with 40000 IU vitamin D for 6 months had no significant effect on depressive symptom scores, when compared with placebo

(54). A recent meta-analysis, which included nine trials with a total of 4923 participants, concluded that no significant reduction in depression was seen after vitamin D supplementation (45). In a double-blinded, placebo controlled prospective trial, involving 489 postmenopausal elderly women, there was no effect of hormone therapy and calcitriol, either individually or in combination, on depression (55). Supplementation with 400 IU of vitamin D combined with 1,000 mg of elemental calcium on measures of depression in a randomized, double-blinded US trial comprising 36,282 postmenopausal women did not affect depression symptoms (56). However, contrastingly, some studies have reported a beneficial effect of vitamin D supplementation on depression (23). For instance, a trial conducted in elderly patients with MDD showed that a single 300-kIU dose of vitamin D results in a decreased score of depression (57); whilst in a study of 441 obese subjects, it was indicated that supplementation with 20.000 or 40.000 IU vitamin D per week for 1 year ameliorates depression symptoms (23). Congruent with previous meta-analyses (58, 59), and a RCT study (23), our study found a significant reduction in depressive symptoms after 3 months vitamin D supplementation. It is possible that vitamin D is clinically beneficial for individuals who are depressed, but not in healthy participants; thus, variability in depression stage could be considered as an explanatory factor for this finding, but clearly necessitates further investigation. In the current trial, zinc supplementation outperformed vitamin D for improving depression score.

Several biological mechanisms for the beneficial effects of zinc and vitamin D on depression have been proposed (44, 60). 'Neurotrophin hypothesis of depression', mainly based on the inverse association between stress and brain-derived neurotrophic factor (BDNF) levels, is one of the most popular hypotheses in the pathophysiology of depression (61). Expression of BDNF has been reported to be down-regulated in depressed patients, compared to non-depressed matched controls (62), however, it is shown to increase after chronic antidepressant administration (62). In Solati, et al., zinc supplementation resulted in increased serum BDNF levels (13). However, in the present study, after 12 weeks of treatment with zinc, serum levels of BDNF dropped significantly compared to baseline, but were not statistically different as compared to the placebo group. This may conceivably be related to a placebo effect, because the placebo group had a significant reduction in levels of BDNF, while no concurrent reduction in depression was noted. In addition, the regression to mean phenomenon, where if a variable is extreme on its first measurement, it will tend to be closer to the average on its second measurement—and if it is extreme on its second measurement, it will tend to have been closer to the average on its first, may be considered as an alternative explanation for the changes in

BDNF in the zinc and placebo groups. At baseline, the zinc group had a significantly higher level of serum BDNF compare to all other groups. In Ranjbar et al, it was reported that serum levels of BDNF in depressed patients receiving zinc supplement did not increase (51). In the present study, to the best of our knowledge, we are the first to report vitamin D and joint zinc-vitamin D supplementation has no effect on serum BDNF.

Other mechanisms have been proposed to mediate the effects of zinc and vitamin D on depression, including modulation of the hypothalamus–pituitary–adrenal (HPA) axis (63). Zinc and vitamin D, an anti-inflammatory element, help to maintain endocrine homeostasis and regulation of the hippocampal and cortical glutamatergic circuits that subserve affective regulation and cognitive function (64, 65). However, in the current trial, serum levels of cortisol in obese patients with depression receiving zinc, vitamin D or their co-supplementation were not decreased. Several hypotheses exist to explain why no beneficial effects of zinc-vitamin D joint supplementation, compared to zinc or vitamin D individually, reduced depression related symptom. The dose of vitamin D we tested (2000 IU/day) may not have been sufficient to affect the depression score in obese patients. Participants in our study were obese individuals with MDD, who might have an increased need to vitamin D. In addition, normal status in zinc at baseline and continuity of vitamin D deficiency at study cessation in the zinc-vitamin D group could explain this finding.

The current trial has several strengths. Mood disorders, particularly depression, are among the leading causes of morbidity in obese subjects (4, 5). Current modalities for treatment of depression are insufficient and expensive (15, 16), and the prevalence of zinc and vitamin D insufficiency is high (66, 67). Furthermore, our study was conducted on patients with depressive symptoms with limited physical activity levels and sun exposure, which, in turn, would contribute to vitamin D insufficiency (53). This study highlights the need for adequately powered randomized clinical trials to establish whether there is a causal relation between zinc, vitamin D status and depression severity in overweight/obese subjects. We believe that this study is the first clinical trial to quantitatively evaluate the joint effects of zinc and vitamin D monotherapy on serum BDNF, cortisol levels and depression severity in overweight/obese subjects with depressive symptoms. In light of the current evidence represents a valuable addition to the current findings on the efficacy of zinc and vitamin D monotherapy on depression symptoms.

However, there are some limitations to the present RCT which must be considered. Firstly, the study population was comprised of obese/overweight subjects with BDI-II≥10 who received zinc, vitamin D supplementation in the absence of anti-depressant medications, thus, these findings may not be generalizable to severely depressed subjects already prescribed anti-depressant medications. Second, our sample size calculation was based on the variability in depression score, and may not have been adequate for the analysis of serum cortisol as a secondary outcome. Third, serum cortisol levels were reported in this trial, however, it is arguable that urine measurement is the preferred method for cortisol assessment. Furthermore, daily physiological and behavioral rhythms including sleep, and body temperature can significantly influence cortisol concentration, which we could not control in the present study. Finally, in the current trial, despite the randomization, serum zinc and vitamin D level at baseline were unequal, however, these variables were adjusted accordingly in the applied analysis.

Supplementation with zinc, vitamin D or joint zinc–vitamin D can improve BDI-II score in obese patients with depressive symptoms. However, the zinc, vitamin D and depression improvements appear to be independent from serum cortisol and BDNF. To confirm the veracity of the findings of present trial, further trials with longer durations and larger sample sizes are needed.

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Author statement contributor:

The authors' responsibilities were as follows: SY, AE and SJ: designed the project, SS and SY wrote the first draft of the manuscript; AM and SJ data analysis and interpreted the data, SJ, AE, CC and MT: revised the subsequent drafts for important intellectual content, and approved the final version of the manuscript to be published.

Conflict of interest:

SY, SS, AE, AM, MT, CC, and SJ declared that there were no conflicts of interest. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Table 1: characterization of participants at baseline and after 12 week

		Zinc (n=24)	Vitamin D (n=27)	Zinc-vitaminD (n=25)	Placebo (n=22)	P-value‡
Age (years)		38.71±7.16	38.28±7.28	38.93±5.39	37.31±7.12	0.823
Blood pressure systolic	**Baseline**	121.20±12.72	121.61±13.81	119.26±14.07	116.79±11.75	0.625
	After 12 week	117.40±11.28	118.00±15.76	114.73±14.66	118.17±12.58	0.790
	P-value^{Ł}	0.117	<0.0001	<0.0001	0.613	
Blood pressure diastolic	**Baseline**	80.68±7.91	84.58±9.79	80.30±10.80	80.21±6.07	0.882
	After 12 week	81.55±7.48	81.96±10.00	78.73±11.94	79.08±10.92	0.127
	P-value^{Ł}	0.055	0.014	<0.0001	0.212	
Weight (Kg)	**Baseline**	86.40±10.13	90.42±16.51	86.31±14.36	87.20±12.86	0.625
	After 12 week	86.62±9.89	90.61±16.01	85.42±14.88	87.67±12.62	0.493
	P-value^{Ł}	0.884	0.636	0.284	0.770	
BMI(kg/ m^2)	**Baseline**	29.08±2.96	30.59±4.08	29.59±3.64	30.11±3.68	0.428
	After 12 week	29.14±2.99	30.63±3.93	29.38±3.48	30.13±3.73	0.373
	P-value^{Ł}	0.866	0.717	0.154	0.850	
Gender(male/female)		26/8	27/7	24/9	26/6	0.760
Waist circumference	**Baseline**	103.63±5.84	106.91±11.39	105.25±8.95	103.84±8.58	0.513
	After 12 week	103.14±6.38	105.33±11.69	103.85±9.20	104.43±9.53	0.846
	P-value^{Ł}	<0.0001	<0.0001	<0.0001	<0.0001	

Note: BMI=Body Mass Index

‡: Values are analyzed by one-way analysis of variance; Ł: Values are analyzed by paired-samples T test

values are mean ± SD

Table 2: change in BDI-II, serum BDNF and cortisol level from baseline to 12 weeks

	Zinc (mean) (95% CI)	Vitamin D (mean) (95% CI)	Zinc-vitaminD(mean) (95% CI)	Placebo (mean) (95% CI)	P-value ǂ	ß
BDI-II score	-7.02 (-9.13, -4.74)	-3.87 (-6.15, -1.59)	-7.62 (-10.63, -4.61)	-0.76 (-3.05, 1.52)	0.0001*	0.969
BDNF	-0.19 (-0.36, -0.02)	-0.11 (-0.28, 0.05)	0.19 (-0.02, 0.41)	-0.17 (-0.34, -0.003)	0.08	0.589
Cortisol	-1.37 (-3.43, 0.67)	-1.66 (-3.71, 0.38)	-0.82 (-3.52. 1.88)	-1.19 (-3.29, 0.90)	0.974	0.056

BDI-II: Beck Depression Score-II
ǂ: Values are analyzed by ANCOVA (adjusted by baseline serum zinc, vitamin D, cortisol and BDNF and beck depression score);
*Significant differences between zinc and placebo groups, vitamin D and placebo groups, zinc-vitamin D and placebo groups, zinc and vitamin D groups
values are mean ± SD

Table 3: serum zinc, vitamin D, cortisol and BDNF, at baseline and after 12 week base on study groups.

		Zinc	Vitamin D	Zinc-vitamin D	Placebo	P-value ǂ	ß
Zinc µg/dl	Baseline	57.13±21.91	60.25±17.74	115.37±22.45	62.48±19.01	>0.001*	
	After 12 week	77.03±47.26	58.60±13.30	127.90±38.65	56.80±14.36	>0.001**	0.969
	P-value Ɨ	0.008	0.588	0.068	0.213		
Vitamin D ng/ml	Baseline	15.86±9.03	26.07±13.27	10.44±5.23	20.51±10.43	>0.001***	
	After 12 week	14.00±8.06	36.29±11.28	17.93±7.28	16.85±10.07	>0.001****	1.000
	P-value Ɨ	0.141	>0.001	>0.001	>0.001		

ǂ: Values are analyzed by ANCOVA (adjusted by baseline serum zinc, vitamin D); Ɨ: Values are analyzed by paired-samples T test

*Significan differences between zinc-vitamin D and placebo groups, zinc-vitamin D and zinc groups, zinc-vitamin D and vitamin D groups

** Significan differences between zinc-vitamin D and placebo groups, zinc-vitamin D and vitamin D groups, zinc and placebo groups, zinc and vitamin D groups.

*** significant differences between vitamin D and zinc groups, zinc- vitamin D and placebo groups, zinc- vitamin D and vitamin D groups.

**** significant differences between zinc-vitamin D and vitamin D groups, zinc-vitamin D and placebo groups, zinc-vitamin D and zinc groups, vitamin D and zinc groups, vitamin D and placebo groups.

Flow diagram1: depicting progress through different phases of the clinical trial