

Effects of calcium–vitamin D co-supplementation on glycaemic control, inflammation and oxidative stress in gestational diabetes: a randomised placebo-controlled trial

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Received: 20 February 2014 / Accepted: 19 May 2014 / Published online: 25 June 2014
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Abstract

Aims/hypothesis This study was designed to assess the effects of calcium and vitamin D supplementation on the metabolic status of pregnant women with gestational diabetes mellitus (GDM).

Methods This randomised placebo-controlled trial was performed at maternity clinics affiliated to Kashan University of Medical Sciences, Kashan, Iran. Participants were 56 women with GDM at 24–28 weeks' gestation (18 to 40 years of age). Subjects were randomly assigned to receive calcium plus vitamin D supplements or placebo. All study participants were blinded to group assignment. Individuals in the calcium–vitamin D group ($n=28$) received 1,000 mg calcium per day and a 50,000 U vitamin D₃ pearl twice during the study (at study baseline and on day 21 of the intervention), and those in the placebo group ($n=28$) received two placebos at the mentioned times. Fasting blood samples were taken at study baseline and after 6 weeks of intervention.

Results The study was completed by 51 participants (calcium–vitamin D $n=25$, placebo $n=26$). However, as the analysis was based on an intention-to-treat approach, all 56 women with GDM (28 in each group) were included in the final analysis. After the administration of calcium plus vitamin D supplements, we observed a significant reduction in fasting plasma glucose (-0.89 ± 0.69 vs $+0.26\pm 0.92$ mmol/l,

$p<0.001$), serum insulin levels (-13.55 ± 35.25 vs $+9.17\pm 38.50$ pmol/l, $p=0.02$) and HOMA-IR (-0.91 ± 1.18 vs $+0.63\pm 2.01$, $p=0.001$) and a significant increase in QUICKI ($+0.02\pm 0.03$ vs -0.002 ± 0.02 , $p=0.003$) compared with placebo. In addition, a significant reduction in serum LDL-cholesterol (-0.23 ± 0.79 vs $+0.26\pm 0.74$ mmol/l, $p=0.02$) and total cholesterol: HDL-cholesterol ratio (-0.49 ± 1.09 vs $+0.18\pm 0.37$, $p=0.003$) and a significant elevation in HDL-cholesterol levels ($+0.15\pm 0.25$ vs -0.02 ± 0.24 mmol/l, $p=0.01$) was seen after intervention in the calcium–vitamin D group compared with placebo. In addition, calcium plus vitamin D supplementation resulted in a significant increase in GSH ($+51.14\pm 131.64$ vs -47.27 ± 203.63 μ mol/l, $p=0.03$) and prevented a rise in MDA levels ($+0.06\pm 0.66$ vs $+0.93\pm 2.00$ μ mol/l, $p=0.03$) compared with placebo.

Conclusions/interpretation Calcium plus vitamin D supplementation in women with GDM had beneficial effects on their metabolic profile.

Trial registration www.irct.ir IRCT201311205623N11

Funding The study was supported by a grant (no. 92110) from Kashan University of Medical Sciences.

Keywords Calcium · Gestational diabetes · Pregnant women · Supplementation · Vitamin D

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Abbreviations

25(OH)D	25-Hydroxyvitamin D
FPG	Fasting plasma glucose
GDM	Gestational diabetes mellitus
GSH	Total glutathione
HOMA-B	Homeostatic model assessment–beta cell function
Hs-CRP	High-sensitivity C-reactive protein
LOCF	Last observation carried forward
MDA	Malondialdehyde
TAC	Total antioxidant capacity

Introduction

Gestational diabetes mellitus (GDM), a pregnancy complication, is characterised by carbohydrate intolerance and metabolic disorders [1]. Approximately 7% of all pregnancies in the USA are affected by GDM, but the prevalence ranges from 1% to 14% of all pregnancies in the world depending on the population studied and the diagnostic criteria used [2]. Overall, 4.7% of pregnant women in Iran are affected by this condition [3]. Various factors, including older age at the time of the first pregnancy, stressful life conditions and a sedentary lifestyle along with less physical activity, inappropriate diet and high-energy food intake, have been reported to increase the risk [4]. GDM is associated with insulin resistance, vascular dysfunction, vascular disease, macrosomia, neonatal hypoglycaemia, hyperbilirubinaemia [5], Caesarean section, pre-eclampsia and preterm delivery [6].

Current therapies for GDM include a low-glycaemic index diet, carbohydrate restriction [7], the use of some oral hypoglycaemic agents [8] and insulin therapy [9]. A few recent studies have shown that supplementation with calcium [10] and vitamin D [11] in patients with GDM might affect pregnancy outcomes. Although the combined effects of calcium and vitamin D supplementation on glucose homeostasis and biomarkers of oxidative stress have not been examined in patients with GDM, some studies have reported the effects of single vitamin D supplementation on metabolic profiles and oxidative stress in these patients [12]. Our previous study in patients with GDM showed that vitamin D supplementation resulted in improved insulin function and decreased concentrations of total cholesterol and LDL-cholesterol after 6 weeks [12]. Calcium and vitamin D have been hypothesised to act jointly rather than independently. Previous reports have shown that joint supplementation is much more efficient in influencing metabolic profiles than single calcium or vitamin D supplementation. Harinarayan et al [13] observed improvement in pancreatic beta cell function (HOMA-B) after supplementation with 10,000 U/day vitamin D and 1,000 mg/day calcium in vitamin D-deficient non-diabetic subjects after 8 weeks. However, a 3 month supplementation with vitamin D (daily dose of 3,533 U, increased to 8,533 U after the first five participants) and 530 mg elemental calcium per day did not affect insulin resistance in overweight women with polycystic ovary syndrome (PCOS) [14]. Requirement for both vitamin D and calcium is increased in pregnancy. Therefore, insufficient nutritional status during this important period of life might increase the risk of GDM. Calcium and vitamin D supplementation might affect metabolic profiles and oxidative stress through their effects on cell cycle regulation [15], activation of antioxidant enzymes [16] and suppression of parathyroid hormone (PTH) [17]. We are aware of no studies of the effect of joint calcium–vitamin D supplementation on insulin function, lipid profiles, inflammatory factors and

biomarkers of oxidative stress in GDM. This study was therefore carried out to investigate the effects of calcium plus vitamin D supplementation on the metabolic status of pregnant women with GDM.

Methods

Participants This randomised placebo-controlled trial was conducted in Kashan, Iran, during September 2013 to November 2013. For estimating sample size, we considered type 1 (α) and type 2 errors (β) of 0.05 and 0.20 (power=80%), respectively, and serum insulin levels as a key variable. On the basis of a previous study [18], the SD of serum insulin was 32.2 pmol/l, and the difference in mean (d) of insulin levels was 25.8 pmol/l. We reached the sample size of 25 participants for each group using the suggested formula for parallel clinical trials. In this study, we included pregnant women aged 18–40 years who had been diagnosed with GDM by a ‘one-step’ 2 h 75 g OGTT at 24–28 weeks’ gestation. Gestational age was assessed from the date of the last menstrual period and concurrent clinical assessment. Pregnant women without a previous diagnosis of glucose intolerance were screened. GDM was diagnosed using the criteria of the ADA [19]: women whose plasma glucose met one of the following criteria were considered to have GDM: fasting ≥ 5.1 mmol/l; 1 h ≥ 10 mmol/l; 2 h ≥ 8.5 mmol/l. A total of 950 pregnant women attending maternity clinics affiliated to Kashan University of Medical Sciences, Kashan, Iran, were screened for GDM. Of these, 56 met the inclusion criteria (886 women were excluded because they did not have GDM, and eight women were not included because of a diagnosis of GDM class A2, which needed insulin therapy: fasting plasma glucose (FPG) > 5.8 mmol/l and 2 h postprandial blood sugar > 6.7 mmol/l). Participants with premature preterm rupture of the membrane, placenta abruption, pre-eclampsia, eclampsia, chronic hypertension, hypothyroidism, urinary tract infection, kidney or liver diseases or stressful life conditions or who were smokers or using oestrogen therapy were not included in the study. We excluded those who were required to start insulin therapy during the intervention (FPG > 5.8 mmol/l and 2 h postprandial blood sugar > 6.7 mmol/l). A total of 56 pregnant women were recruited; after stratification for preintervention BMI (< 30 and ≥ 30 kg/m²) and weeks of gestation (< 26 or ≥ 26), they were randomly assigned to calcium plus vitamin D supplements ($n=28$) or placebo ($n=28$) for 6 weeks. Random assignment was achieved by using computer-generated random numbers. Randomisation and allocation were concealed from the researcher and participants until the main analyses were completed. A trained midwife at the maternity clinic, who was not blinded to the intervention, carried out the randomised allocation sequence, enrolled participants, and assigned participants to interventions. The study

was conducted according to the guidelines laid down in the Declaration of Helsinki. It was approved by the ethics committee of Kashan University of Medical Sciences and registered on the Iranian Registry of Clinical Trials website (IRCT registration no. 201311205623N11). All participants provided written informed consent before recruitment.

Study design Participants were randomly assigned to take calcium–vitamin D supplements or placebo. Individuals in the calcium–vitamin D group received 1,000 mg calcium carbonate per day plus 50,000 U vitamin D₃ pearl twice during the study: at the study baseline and on day 21 of the intervention. Individuals in the placebo group received separate placebos for calcium (daily) and for vitamin D (twice during the study: at the study baseline and on day 21 of the intervention). The calcium supplement and its placebo were manufactured by Tehran Shimi Pharmaceutical Company (Tehran, Iran). Vitamin D and its placebo were manufactured by Dana Pharmaceutical Company (Tabriz, Iran) and Barij Essence Pharmaceutical Company (Kashan, Iran). The duration of the intervention was 6 weeks; however, vitamin D and its placebo were given only twice during the 6 weeks. The appearance of the placebo tablets and capsules (i.e. colour, shape, size and packaging) was identical with the calcium tablets and vitamin D₃ capsules. Calcium and vitamin D and their placebos were packed in identical packages and coded by the producer to guarantee blinding. Quality control of the calcium and vitamin D supplements was carried out in the laboratory of the Food and Drug Administration in Tehran, Iran by enzymatic and HPLC methods. After quality control, we found that the amount of calcium and cholecalciferol in the prescribed supplements was in the range 950–1,200 mg and 47,500–52,500 U, respectively. Participants were asked not to alter their routine physical activity or usual dietary intakes throughout the study and not to consume any supplements other than the one provided by the investigators. All participants were also consuming 400 µg/day folic acid from the beginning of pregnancy and 60 mg/day ferrous sulphate from the second trimester. Compliance with the calcium plus vitamin D supplementation was assessed by quantifying serum calcium and vitamin D levels. As serum calcium levels cannot completely reflect dietary or supplemental calcium intake, participants were also asked to bring the medication containers, and compliance was double checked by counting unused tablets. Dietary intakes of participants throughout the intervention were assessed by means of 3 day dietary records. The dietary records were based on estimated values in household measurements. To obtain nutrient intakes of participants based on these 3 day food diaries, we used Nutritionist IV software (First Databank, San Bruno, CA, USA) modified for Iranian foods.

Assessment of variables Data on prepregnancy weight and height (measured values) were taken from the records of the

pregnant women in the clinic. A trained midwife at the maternity clinic performed the anthropometric measurements at study baseline and 6 weeks after the intervention. Body weight was measured to the nearest 0.1 kg, with participants in an overnight-fasted state, without shoes and in minimal clothing, using digital scales (Seca, Hamburg, Germany). Height was measured using a non-stretched tape measure (Seca) to the nearest 0.1 cm. BMI was calculated as weight in kg divided by height in m².

At baseline and after 6 weeks of the intervention, 10 ml venous blood samples were taken at the Kashan University of Medical Sciences reference laboratory after overnight fasting. FPG was measured on the day of blood collection. Blood samples were centrifuged immediately (Hettich D-78532, Tuttlingen, Germany) at 1,465 g for 10 min to separate serum. Serum lipid profiles were also quantified on the day of blood collection. Then the samples were stored at –70°C before analysis at the Kashan University of Medical Sciences reference laboratory. Serum 25-hydroxyvitamin D [25(OH)D] concentrations were assayed using a commercial ELISA kit (IDS, Boldon, UK). The inter- and intra-assay CVs for serum 25(OH)D assays ranged from 4.9% to 7.1%. Commercial kits were used to measure FPG, serum calcium, cholesterol, triacylglycerol, LDL-cholesterol and HDL-cholesterol concentrations (Pars Azmun, Tehran, Iran). The intra- and inter-assay CVs for FPG were 1.9% and 3.2%, respectively. All inter- and intra-assay CVs for lipid profile measurements were less than 5%. Serum insulin levels were assayed with an ELISA kit (DiaMetra, Milano, Italy). The intra- and inter-assay CVs for serum insulin were 3.1% and 5.9%, respectively. HOMA-IR, HOMA-B and QUICKI were calculated on the basis of suggested formulas. Serum high-sensitivity C-reactive protein (hs-CRP) was quantified using an ELISA kit (LDN, Nordhorn, Germany) with intra- and inter-assay CVs of 2.6% and 4.5%, respectively. Plasma NO concentration was determined by the Griess method. Plasma total antioxidant capacity (TAC) was assessed by the ferric-reducing ability of plasma (FRAP) method developed by Benzie and Strain [20]. Plasma total glutathione (GSH) was determined using the method of Beutler et al [21], and plasma malondialdehyde (MDA) using the thiobarbituric acid reactive substance (TBARS) spectrophotometric test. CVs for plasma TAC, GSH and MDA were 0.9%, 2.45% and 3.5%, respectively. Measurements of vitamin D, calcium, glucose, lipid, insulin, TAC, GSH and MDA were performed in a blinded fashion, in duplicate, in pairs (pre-/post-intervention) at the same time, in the same analytical run, and in random order to reduce systematic error and interassay variability.

Statistical analysis We used the Kolmogorov–Smirnov test to examine the normal distribution of variables. Log transformation was applied for non-normally distributed variables. The analyses were performed on the basis of an intention-to-treat

approach. Missing values were dealt with using the last observation carried forward (LOCF) method. The independent-sample Student's *t* test was used to detect differences in general characteristics and dietary intakes between the two groups. To determine the effects of calcium plus vitamin D supplementation on glucose metabolism, lipid profiles, inflammatory factors and biomarkers of oxidative stress, we used one-way repeated measures ANOVA. In this analysis, the treatment (calcium plus vitamin D vs placebo) was regarded as a between-subject factor, and time with two time points (baseline and week 6 of the intervention) as a within-subject factor. To assess if the magnitude of the change in dependent variables depended on the baseline values, maternal age and baseline BMI, we controlled all analyses for baseline values, maternal age and baseline BMI to avoid the potential bias that might have resulted. These analyses were performed using ANCOVA. $p < 0.05$ was considered significant. All statistical analyses were performed using SPSS version 17 (SPSS, Chicago, IL, USA).

Results

Three individuals in the calcium plus vitamin D group were excluded: intrauterine fetal death (IUFD) ($n=1$) and hospitalisation ($n=2$). Two women in the placebo group were also excluded: placenta abruption ($n=1$) and insulin therapy ($n=1$). Finally, 51 participants (calcium plus vitamin D [$n=25$] and placebo [$n=26$]) completed the trial (Fig. 1).

However, as the analysis was based on an intention-to-treat approach, all 56 women (28 in each group) were included in the final analysis. For those who completed the trial ($n=51$), the tablet and capsule counts suggested 100% adherence in both groups.

The mean \pm SD age, prepregnancy weight and BMI of study participants was 29.8 ± 6.3 years, 75.9 ± 13.4 kg and 29.9 ± 4.6 kg/m², respectively. The mean gestational age at the study baseline was 25.6 ± 1.3 weeks. Baseline and end-of-trial means of weight and BMI were not significantly different between the two groups (Table 1).

Based on 3 day dietary records obtained throughout the intervention, no statistically significant difference was seen between the two groups in terms of dietary intake of energy, carbohydrates, proteins, fats, saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, cholesterol, total dietary fibre, magnesium, calcium, manganese, vitamin D, vitamin C and selenium (Table 2).

Baseline values of FPG, HOMA-B, QUICKI, GSH and MDA were significantly different between the two groups. Calcium plus vitamin D supplementation, compared with placebo, led to a significant rise in serum 25(OH)D levels ($+48.19 \pm 46.64$ vs $+1.75 \pm 15.36$ nmol/l, $p < 0.001$) and a slight increase in serum calcium concentrations ($+0.17 \pm 0.48$ vs -0.03 ± 0.34 mmol/l, $p = 0.08$) (Table 3). In addition, after the administration of calcium plus vitamin D supplements, we observed a significant reduction in FPG (-0.89 ± 0.69 vs $+0.26 \pm 0.92$ mmol/l, $p < 0.001$), serum insulin levels (-13.55 ± 35.25 vs $+9.17 \pm 38.50$ pmol/l, $p = 0.02$) and HOMA-IR (-0.91 ± 1.18 vs $+0.63 \pm 2.01$, $p = 0.001$) and a significant

Fig. 1 Summary of patient flow diagram. Individuals in the calcium plus vitamin D group received 1,000 mg calcium carbonate per day plus 50,000 U vitamin D₃ pearl twice during the study (at study baseline and on day 21 of the intervention), and those in the placebo group received placebo for calcium each day and placebo for vitamin D twice during the study (at study baseline and on day 21 of the intervention)

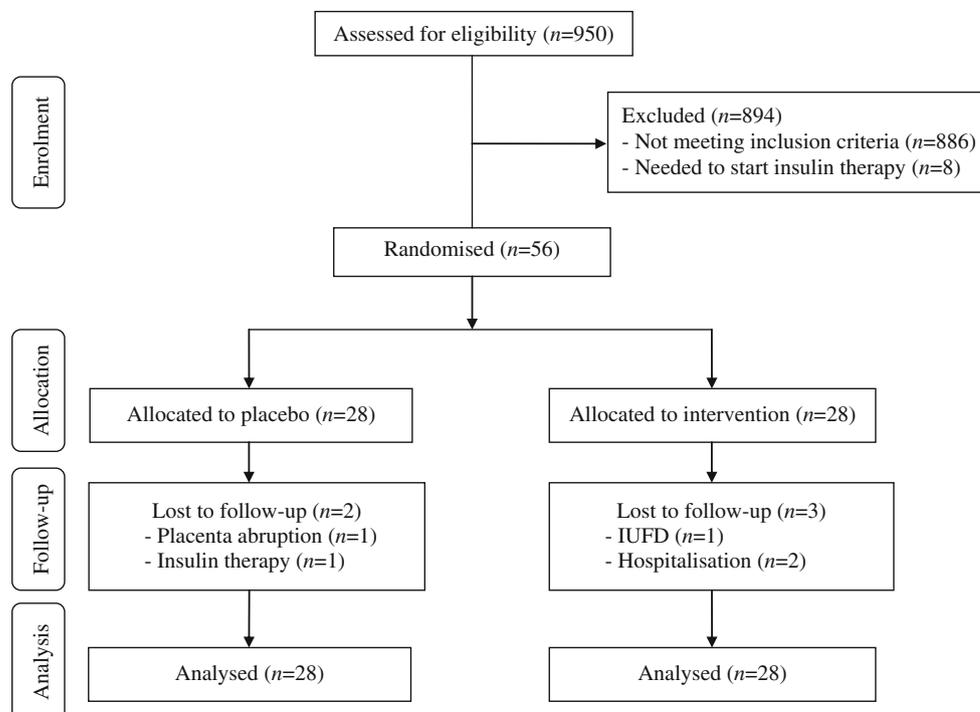


Table 1 General characteristics of pregnant women with GDM who received either calcium plus vitamin D supplements or placebo

Characteristic	Placebo group ^a (n=28)	Calcium plus vitamin D group ^b (n=28)
Maternal age (years)	30.8±6.6	28.7±6.0
Height (cm)	159.9±4.4	158.1±4.6
Prepregnancy weight (kg) ^c	69.5±12.1	67.9±12.2
Weight at study baseline (kg)	78.2±13.6	73.6±13.0
Weight at end of trial (kg)	79.9±13.3	75.5±13.2
Weight change (kg)	1.7±1.4	1.9±1.6
Prepregnancy BMI (kg/m ²) ^c	27.1±4.3	27.1±4.4
BMI at study baseline (kg/m ²)	30.5±4.6	29.4±4.6
BMI at end of trial (kg/m ²)	31.2±4.5	30.2±4.7
BMI change (kg/m ²)	0.7±0.6	0.8±0.7

All values are means ± SD

Statistical significance was determined using an independent *t* test

^a Received placebos for calcium daily and for vitamin D twice during the study: at study baseline and on day 21 of the intervention

^b Received 1,000 mg calcium carbonate daily plus 50,000 U vitamin D₃ twice during the study: at study baseline and on day 21 of the intervention

^c Based on measured weight and height in the participants' records in the maternity clinics

increase in QUICKI (+0.02±0.03 vs -0.002±0.02, *p*=0.003) compared with placebo. A significant reduction in serum LDL-cholesterol (-0.23±0.79 vs +0.26±0.74 mmol/l, *p*=0.02) and total cholesterol: HDL-cholesterol ratio (-0.49±1.09 vs +0.18±0.37, *p*=0.003) and a significant elevation in HDL-cholesterol (+0.15±0.25 vs -0.02±0.24 mmol/l, *p*=0.01) were seen after intervention in the calcium–vitamin D group compared with placebo. Furthermore, supplementation resulted in a significant increase in plasma GSH (+51.14±131.64 vs -47.27±203.63 μmol/l, *p*=0.03) and prevented the rise in plasma MDA levels (+0.06±0.66 vs +0.93±2.00 μmol/l, *p*=0.03). We did not find any significant effect of calcium plus vitamin D supplementation on HOMA-B, serum total cholesterol, triacylglycerol, hs-CRP, NO and plasma TAC.

When we adjusted the analyses for baseline values, no significant changes in our findings were observed except for serum calcium (*p*=0.03), plasma GSH (*p*=0.20) and MDA levels (*p*=0.06) (Table 4). Additional adjustment for age and baseline BMI did not affect our findings.

Discussion

In this study, calcium plus vitamin D supplementation of pregnant women with GDM resulted in improved glycaemic status, a significant decrease in serum LDL-cholesterol, a significant rise in HDL-cholesterol and plasma GSH, and a significant difference in plasma MDA levels, but did not affect

Table 2 Dietary intakes of pregnant women with GDM who received either calcium plus vitamin D supplements or placebo throughout the study

Dietary intake	Placebo group ^a (n=28)	Calcium plus vitamin D group ^b (n=28)
Energy (kJ/day)	10,040±1,396 ^c	10,332±815
Carbohydrates (g/day)	333.3±37.6	338.7±60.4
Protein (g/day)	90.5±14.5	86.8±12.2
Fat (g/day)	87.2±10.7	82.3±14.2
SFAs (g/day)	25.5±4.7	26.5±6.1
PUFAs (g/day)	27.1±7.8	24.1±6.1
MUFAs (g/day)	24.4±5.8	23.6±6.7
Cholesterol (mg/day)	213.2±118.7	192.5±58.4
TDF (g/day)	19.7±4.1	20.4±4.8
Magnesium (mg/day)	301.9±59.4	288.8±69.9
Calcium (mg/day)	1,166.8±191.3	1,145.2±210.8
Manganese (mg/day)	2.4±0.7	2.5±1.0
Vitamin D (μg/day)	2.8±0.9	2.7±0.7
Vitamin C (mg/day)	194.1±92.6	200.7±73.9
Selenium (μg/day)	110.9±20.8	117.3±34.0

All values are mean ± SD

Statistical significance was determined using an independent *t* test

^a Received placebos for calcium daily and for vitamin D twice during the study: at study baseline and on day 21 of intervention

^b Received 1,000 mg calcium carbonate daily plus 50,000 U vitamin D₃ twice during the study: at study baseline and on day 21 of intervention

MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; TDF, total dietary fibre

other lipid profiles, inflammatory factors or plasma TAC. To the best of our knowledge, this is the first study to examine the effects of calcium plus vitamin D supplementation on metabolic status in pregnant women with GDM.

GDM is associated with insulin resistance, increased inflammatory factors and oxidative stress [5]. Elevated circulating levels of inflammatory markers and impaired insulin metabolism in GDM can predict the progression to type 2 diabetes later in life and neonatal complications [22]. Impaired insulin metabolism in women with GDM can result in adverse long-term maternal outcomes, increased perinatal morbidity (e.g. macrosomia, birth trauma, pre-eclampsia) and long-term sequelae in the offspring [5, 6]. In addition, increased inflammatory markers in GDM might predict future development of both metabolic and cardiovascular disease [23]. Our study demonstrated that calcium plus vitamin D supplementation for 6 weeks in pregnant women with GDM led to a significant decrease in FPG, serum insulin levels and HOMA-IR score and a significant rise in QUICKI compared with placebo. Previous studies have reported the effects of single calcium or vitamin D supplementation on glucose metabolism of patients with diabetes and GDM, but the combined effects of calcium and vitamin D supplementation in patients with GDM

Table 3 Metabolic profiles, inflammation and biomarkers of oxidative stress at study baseline and after a 6-week intervention in pregnant women with GDM who received either calcium plus vitamin D supplements or placebo

Variable	Placebo group ^a (n=28)			Calcium plus vitamin D group ^b (n=28)			p value ^c
	Week 0	Week 6	Change	Week 0	Week 6	Change	
Calcium (mmol/l)	2.03±0.32 ^d	2.00±0.38	-0.03±0.34	2.03±0.44	2.20±0.34	0.17±0.48	0.08
Vitamin D (nmol/l)	49.05±34.30	50.80±35.48	1.75±15.36	43.11±28.17	91.30±54.60*	48.19±46.64	<0.001
FPG (mmol/l)	4.42±0.59	4.68±1.06	0.26±0.92	5.14±0.71	4.25±0.64*	-0.89±0.69	<0.001
Insulin (pmol/l)	88.39±50.13	97.56±64.43	9.17±38.50	77.87±36.51	64.32±28.73	-13.55±35.25	0.02
HOMA-IR	2.93±1.66	3.56±2.69	0.63±2.01	2.93±1.34	2.02±0.89*	-0.91±1.18	0.001
HOMA-B	63.32±39.94	65.29±44.84	1.97±28.51	48.45±26.87	47.68±23.87	-0.77±29.66	0.63
QUICKI	0.33±0.03	0.33±0.05	-0.002±0.02	0.33±0.01	0.35±0.03*	0.02±0.03	0.003
Total cholesterol (mmol/l)	5.11±1.20	5.33±1.47	0.22±0.96	5.10±1.01	5.06±1.20	-0.04±1.01	0.33
Triacylglycerol (mmol/l)	2.09±0.69	2.05±0.68	-0.04±0.63	2.02±0.78	2.14±0.84	0.12±0.58	0.36
LDL-cholesterol (mmol/l)	2.79±0.88	3.05±1.08	0.26±0.74	2.94±0.81	2.71±0.87	-0.23±0.79	0.02
HDL-cholesterol (mmol/l)	1.36±0.44	1.34±0.44	-0.02±0.24	1.22±0.27	1.37±0.33*	0.15±0.25	0.01
Total: HDL-cholesterol ratio	3.93±0.86	4.11±0.80*	0.18±0.37	4.32±1.21	3.83±0.96*	-0.49±1.09	0.003
hs-CRP (ng/ml)	6,451.03±3,924.81	6,244.83±4,192.56	-206.20±4,006.65	7,391.83±4,693.09	7,384.93±3,692.78	-6.90±4006.65	0.83
NO (μmol/l)	54.04±28.65	51.69±28.40	-2.35±28.65	60.39±32.56	66.48±29.41	6.09±34.07	0.32
TAC (mmol/l)	724.71±176.51	789.39±176.47*	64.68±128.19	702.08±190.66	751.34±129.29	49.26±140.80	0.67
GSH (μmol/l)	761.69±383.11	714.42±365.83	-47.27±203.63	570.91±97.73	622.05±127.85	51.14±131.64	0.03
MDA (μmol/l)	2.69±1.42	3.62±1.83*	0.93±2.00	2.99±0.53	3.05±0.72	0.06±0.66	0.03

^a Received placebos for calcium daily and for vitamin D twice during the study: at study baseline and on day 21 of intervention^b Received 1,000 mg calcium carbonate daily plus 50,000 U vitamin D₃ twice during the study: at study baseline and on day 21 of intervention^c Obtained from repeated measures ANOVA^d All values are mean ± SD. Baseline values of FPG, HOMA-B, QUICKI, GSH and MDA were significantly different between the two groups*Different from Week 0, $p < 0.05$

Table 4 Adjusted changes in metabolic variables in pregnant women with GDM who received either calcium plus vitamin D supplements or placebo

Variable	Placebo group ^a (n=28)	Calcium plus vitamin D group ^b (n=28)	p value ^c
Vitamin D (nmol/l)			
Model 1 ^d	1.84±6.61 ^f	48.07±6.61	<0.001
Model 2 ^e	1.34±6.73	48.54±6.73	<0.001
Calcium (mmol/l)			
Model 1	-0.03±0.06	0.16±0.06	0.03
Model 2	-0.03±0.07	0.16±0.07	0.08
FPG (mmol/l)			
Model 1	0.11±0.16	-0.75±0.16	0.001
Model 2	0.23±0.15	-0.87±0.15	<0.001
Insulin (pmol/l)			
Model 1	10.26±6.84	-14.58±6.84	0.01
Model 2	10.38±7.08	-14.7±7.08	0.01
HOMA-IR			
Model 1	0.65±0.32	-0.91±0.31	0.001
Model 2	0.59±0.30	-0.93±0.30	0.001
HOMA-B			
Model 1	4.25±5.24	-3.06±5.24	0.33
Model 2	3.29±5.49	-2.10±5.49	0.49
QUICKI			
Model 1	-0.003±0.006	0.02±0.006	0.002
Model 2	-0.003±0.006	0.02±0.006	0.003
Total cholesterol (mmol/l)			
Model 1	0.22±0.18	-0.03±0.18	0.32
Model 2	0.20±0.18	-0.02±0.18	0.37
Triacylglycerol (mmol/l)			
Model 1	-0.02±0.10	0.10±0.10	0.39
Model 2	-0.03±0.11	0.10±0.11	0.40
LDL-cholesterol (mmol/l)			
Model 1	0.23±0.14	-0.21±0.14	0.03
Model 2	0.24±0.14	-0.21±0.14	0.03
HDL-cholesterol (mmol/l)			
Model 1	-0.008±0.04	0.13±0.04	0.04
Model 2	-0.02±0.04	0.14±0.04	0.01
Total: HDL-cholesterol ratio			
Model 1	0.09±0.12	-0.40±0.12	0.009
Model 2	0.17±0.15	-0.48±0.15	0.006
hs-CRP (ng/ml)			
Model 1	-399.20±576.98	186.02±576.98	0.47
Model 2	-190.22±680.30	-22.62±680.30	0.86
NO (μmol/l)			
Model 1	-4.19±4.95	7.93±4.95	0.09
Model 2	-2.55±6.06	6.29±6.06	0.31
TAC (mmol/l)			
Model 1	69.36±21.21	44.57±21.21	0.41
Model 2	63.80±26.05	50.12±26.05	0.71

Table 4 (continued)

Variable	Placebo group ^a (n=28)	Calcium plus vitamin D group ^b (n=28)	p value ^c
GSH (μmol/l)			
Model 1	-27.45±31.68	31.32±31.68	0.20
Model 2	-55.23±32.49	59.10±32.49	0.01
MDA (μmol/l)			
Model 1	0.84±0.25	0.15±0.25	0.06
Model 2	0.91±0.28	0.07±0.28	0.04

^a Received placebos for calcium daily and for vitamin D twice during the study: at study baseline and on day 21 of intervention

^b Received 1,000 mg calcium carbonate daily plus 50,000 U vitamin D₃ twice during the study: at study baseline and on day 21 of intervention

^c Obtained from analysis of covariance

^d Adjusted for baseline values

^e Additionally adjusted for maternal age and baseline BMI

^f All values are mean ± SD

have not been assessed. In line with our study, Harinarayan et al [13] showed that 1,000 mg/day calcium and 9,570 U/day vitamin D supplementation in vitamin D-deficient non-diabetic subjects for 2 months resulted in improved FPG and HOMA-B levels. Decreased FPG levels were also seen after the long-term administration of 500 mg calcium plus 700 U vitamin D per day in non-diabetic adults [24]. In our previous study, in pregnant women with GDM, we observed improved glucose metabolism with an intake of 50,000 U vitamin D₃ pearl twice during a 6 week clinical trial [12]. In contrast with our findings, some studies did not observe the effect of calcium or vitamin D supplementation on glucose metabolism. A 3 month supplementation with vitamin D (daily dose of 3,533 U, increased to 8,533 U after the first five participants entered into the study) and calcium (530 mg elemental calcium per day) did not influence insulin function in overweight vitamin D-deficient women with PCOS [14]. It seems that the characteristics of study participants as well as the dosage of supplementation might explain different findings. Beneficial effects of vitamin D supplementation on improved insulin action might result from its effect on calcium and phosphorus metabolism and through upregulation of the insulin receptor genes [25]. Furthermore, vitamin D via the 1,25-dihydroxyvitamin D₃-mediated Ca²⁺ signalling pathway may be involved in the regulation of insulin secretion from the pancreatic beta cell [26].

In the present study, calcium plus vitamin D supplementation in patients with GDM resulted in a significant decrease in serum LDL-cholesterol and total cholesterol: HDL-cholesterol ratio and a significant increase in HDL-cholesterol compared with placebo, but it did not affect serum total cholesterol and triacylglycerol levels. Few studies have examined the combined effects of calcium–vitamin D supplements on lipid profiles. In

agreement with our findings, Major et al [27] found improved lipid profiles in the combined supplementation group (receiving 1,200 mg/day calcium plus 400 U vitamin D) compared with placebo after 15 weeks. Single supplementation with calcium (1–2 g/day) has been shown to lower total cholesterol and LDL-cholesterol concentrations by 5% and increase HDL-cholesterol by 5% [28]. However, some investigators did not find a significant effect of combined calcium–vitamin D or single calcium supplementation on lipid profiles, in either postmenopausal women [29] or obese adults [30]. Different study designs, omitting consideration of baseline levels of dependent variables, and diverse characteristics of study participants might provide some possible reasons for discrepant findings. A mechanism by which calcium intake might potentially exert a lowering effect on circulating lipids is inhibition of the absorption of dietary fatty acids [31]. In addition, calcium supplementation may also result in decreased lipid concentrations through its effect on concentrations of PTH and vitamin D, which also regulate adipocyte activity [17]. Although the exact mechanism by which vitamin D affects lipid concentrations is unknown, it is likely that improved insulin sensitivity might mediate its potential benefit on lipid profiles [29].

Findings from the present study revealed that calcium–vitamin D supplementation in patients with GDM did not affect serum hs-CRP and plasma NO concentrations compared with placebo. In agreement with our study, supplementation with 2 g/day calcium plus 800 U vitamin D had no significant effect on serum hs-CRP levels in patients with colorectal adenoma after 6 months [32]. The same has been found with the consumption of 400 ml/day of milk containing 1,000 mg calcium plus 800 U vitamin D in men aged 50–79 years after 18 months [33]. In contrast, a significant decrease in serum hs-CRP levels was seen in our previous study in healthy pregnant women after they took 400 U vitamin D [34]. In addition, a 9 week supplementation with 500 mg/day calcium plus 80 mg/day aspirin in pregnant women at risk of pre-eclampsia resulted in a significant reduction in serum hs-CRP levels [35]. Different findings might be explained by different study designs, discrepancy in participants' conditions, different dosages of calcium and vitamin D supplementation, and different study durations.

We found that taking calcium–vitamin D supplements led to a significant rise in plasma GSH and prevented the rise in plasma MDA compared with placebo; however, it did not affect plasma TAC. In agreement with our study, Ekici et al [36] have shown increased GSH after consumption of vitamin D₃ plus docosahexaenoic acid in rats, but this did not affect MDA levels. Decreased oxidative DNA damage was also seen after the use of calcium and vitamin D₃ supplements in normal human colorectal mucosa [37]. The exact mechanisms by which calcium and vitamin D supplementation might affect biomarkers of oxidative stress are unknown. Combined

calcium–vitamin D₃ might have a lesser effect on oxidative stress and a greater impact on antioxidants, including GSH levels, than does either calcium or vitamin D alone [16]. In addition, taking a calcium supplement may affect oxidative stress through calcium transport and cell signalling [38].

Some limitations need to be taken into account in the interpretation of our findings. Owing to limited funding, we did not assess the effect of calcium plus vitamin D supplementation on other biomarkers of systemic inflammation or biomarkers of oxidative stress. In addition, we did not assess the effects of calcium and vitamin D supplementation on pregnancy outcomes. However, earlier studies have shown that admissions to neonatal intensive care units were significantly reduced when maternal glycaemic control improved [8]. In addition, management of inflammation through administration of anti-inflammatory drugs during pregnancy significantly regulated ambulatory blood pressure and reduced the incidence of pre-eclampsia, gestational hypertension, preterm delivery and intrauterine growth retardation [39].

Another limitation of this study was loss to follow-up. It must be kept in mind that we used the LOCF method for missing values. LOCF ignores whether the participant's condition was improving or deteriorating at the time of dropout but instead freezes outcomes at the value observed before dropout (i.e. the last observation). This method may introduce bias in the results, and this bias can, according to the circumstances, be in either direction. This might potentially bias the findings toward the null hypothesis for most measures; however, for MDA, where having no change would be an indicator of success of the therapy in the intervention group, this could bias the findings toward a favourable outcome. It is difficult to quantify the magnitude of the effect of the use of LOCF analysis on trial results. This bias is probably not critical in this study because of the small percentage of dropouts.

In conclusion, calcium plus vitamin D supplementation in pregnant women with GDM had beneficial effects on glucose metabolism, lipid profiles and biomarkers of oxidative stress.

Acknowledgements The authors would like to thank the staff of Naghavi and Shaheed Beheshti Clinics (Kashan, Iran) for their assistance in this project.

Funding The study was supported by a grant (no. 92110) from Kashan University of Medical Sciences.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement ZA contributed to conception and design, and analysis and interpretation of data and revising the article. MK contributed to data collection and manuscript drafting. AE contributed to conception and design, and analysis and interpretation of data, and revising the article, and is the guarantor of this work. All authors read and approved the final version of the paper.

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