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Original article

Soy isoflavones and cholecalciferol reduce inflammation, and gut permeability, without any effect on antioxidant capacity in irritable bowel syndrome: A randomized clinical trial

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SUMMARY

Background & aims: Irritable bowel syndrome (IBS) is a common gastrointestinal disorder that is more prevalent in women. Vitamin D deficiency and hormonal disorders are also prevalent in Iranian women, and may influence the severity of clinical outcomes mediated by microinflammation, oxidative stress and intestinal permeability pathways. Our objective was to investigate the effects of co-administration of soy and vitamin D on some inflammatory, antioxidant and gut permeability markers in women with IBS.
Methods: In a randomized clinical trial, women (18–75 years of age) were randomly allocated into four groups to receive soy isoflavones (40 mg/day), cholecalciferol (50,000 IU/15 days), both soy isoflavones and cholecalciferol, or placebo for six weeks. The outcomes were plasma inflammatory markers, antioxidant status and fecal protease activity at week 0 and week 6.
Results: After the intervention, plasma inflammatory markers and fecal protease activity were reduced significantly in all treatment groups compared to the placebo group; however, there was no significant effect on antioxidant status.
Conclusion: This study suggests combined supplementation of soy isoflavones and active vitamin D can improve some biochemical parameters regarding inflammation and intestinal permeability of IBS in women.
Trial registration: Clinical.Trials.gov NCT02026518.

1. Introduction

Irritable bowel syndrome (IBS) is a common gastrointestinal disorder with unknown etiology [1]. There is some evidence that disordered intestinal immune function and intestinal hyperpermeability may be involved in the pathology of IBS [2]. Mounting evidence suggests that sex hormones especially estrogen can influence on the etiology and remission of IBS. The regulation of motor and sensory neurons of gastrointestinal tract can be largely affected by the circulating estrogen level and gonadal disfunction can result in pain and discomfort in women with IBS due to altered intestinal mobility, permeability and immunity [3].

Inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and transcription factors such as nuclear factor-kappa beta (NF-κβ) have a role in the regulation of innate and adaptive immune responses as well as intestinal hyperpermeability in IBS [4,5]. NF-κβ is an important factor in activation of mucosal inflammation, and high levels of TNF-α can trigger the release of inhibitor of kappa B (IκB) and activation of NF-κβ [6]. TNF-α is one of the major pro-inflammatory cytokines that increase epithelial tight junction permeability through the NF-κβ pathway [7]. Increased TNF-α also results in production and release of other pro-inflammatory cytokines that elevate intestinal permeability [8].

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Abbreviations

IBS	irritable bowel syndrome
TNF-α	tumor necrosis factor-alpha
NF-κβ	nuclear factor-kappa beta
IκB	inhibitor kappa B
CD4	cluster of differentiation 4
TAC	total antioxidant capacity
ER-β	estrogen receptor type β
BMI	body mass index
NNFTRI	National Nutrition and Food Technology Research Institute
IU	international unit
MCT	medium chain triglycerides
ELISA	enzyme-linked immunosorbent assay
PBS	phosphate buffer saline
FBS	fetal bovine serum
SD	standard deviation
ANCOVA	analysis of covariance
IL	interleukin
SNPs	single-nucleotide polymorphisms

Studies investigating IBS have shown that low-grade inflammation may trigger symptoms in patients [4]. Moreover, oxidative stress can initiate the entry of luminal contents and activation of CD4 T cells in the intestinal immune system and prolong the inflammatory response [9]; thus, the antioxidant defense system has a critical role in preventing recurrence of symptoms. Total antioxidant capacity (TAC) is a reliable marker of systemic antioxidant defense level [10].

Increased gut permeability is involved in the onset of symptoms of IBS [11]. If pathogens penetrate the leaky tight junctions, a low-grade pro-inflammatory response will initiate and worsen the clinical outcomes of the disorder, and can even lead to chronic inflammation (such as colitis) and cancer [12]. High proteolytic activity is a marker of intestinal hyperpermeability in patients with IBS and has been shown to be increased in diarrhea-type IBS. Fecal serine protease is a newly discussed marker of epithelial permeability in IBS [13].

Recent studies have investigated the effect of cyclic alteration of estrogen activity in female IBS patients on severity and occurrence of clinical outcomes [14]. Soy isoflavones are estrogen-like compounds that mimic the function of estrogen and activate type β estrogen receptors (ER-β) to reduce hyperpermeability [15]. Similarly, the active form of vitamin D modulates the nuclear pathway of ER activation in intestinal smooth muscles [16]. Thus, co-administration of isoflavones with cholecalciferol may reduce the frequency and severity of clinical outcomes by modulating permeability and sensitivity [17].

The present study was conducted to investigate the effect of co-supplementation with soy isoflavone and cholecalciferol on plasma TNF-α, leukocyte NF-κβ activity, plasma TAC and fecal serine protease in women with IBS.

2. Methods and materials

2.1. Study design

Female patients with IBS were recruited according to eligibility criteria (18–75 years of age; registered IBS diagnosis at the Gastrointestinal Disorders Research Institute, Tehran University of Medical Sciences, Tehran, Iran). The study recruitment flow chart is presented in Fig. 1. The Rome III criteria were used to diagnose IBS [18]. The inclusion criteria were diagnosis of IBS (any subtype) and no other intestinal diseases, intestinal infection, history of intestinal surgeries, or regular use of antibiotics, anti-diarrheal or anti-constipation medications, metoclopramide, cisapride, difenoxylate, opium, immune suppressors or anti-inflammatory medications. The exclusion criteria were intake of any soy or vitamin D products in the form of food or medicine, intake of any sugar-free sweeteners two days before and during the clinical study (as they may cause alterations in intestinal permeability), history of breast cancer in the participant or close relatives, pregnancy and lactation, and any type of hormone therapy. Participants with no desire to continue the clinical trial were able to leave the study whenever they wished. All participants who met the eligibility criteria started the study. The study protocol was approved by the Ethics Committee of the National Nutrition and Food Technology Research Institute (NNFTRI), Tehran, Iran (registration number 92-10-10-459). Before starting the intervention, all steps of the study and rights and responsibilities of participants were explained in detail, and a consent form was signed by each participant. Participants were randomly allocated to four study groups (25 equal-size allocation blocks [n = 4]): soy isoflavones and a vitamin D placebo; soy isoflavones and vitamin D; a soy isoflavone placebo and vitamin D placebo; and a soy isoflavone placebo and vitamin D. The intervention period was six weeks. The principal investigator allocated patients to the study groups using sealed envelopes. All researchers were blinded to the allocation procedure. The soy isoflavone supplement contained 10 mg daidzein, 8.5 mg genistein, and 1.5 mg glycetin and was taken twice daily (21st Century Co., USA). The vitamin D supplement contained 50,000 IU active form (cholecalciferol) that was taken once every 14 days (Zahravi co., Iran). The placebos were produced in the same shape and size of each supplement. The soy isoflavone placebo contained 10 mg cornstarch and the vitamin D placebo contained 10 mg medium-chain triglyceride (MCT) oil (Zahravi, Iran). The MCT oil contained 59.4% caprylic acid, 39.6% capric acid, 0.7% caproic acid, 0.2% lauric acid, and 0.1%myristic acid.

Clinical assessments were the primary objectives of this study and the procedure, data, and findings have been reported in detail in a previous published article [17]. Some baseline characteristics of the study groups are presented in Table 1. The effect of co-supplementation with soy isoflavone and cholecalciferol on plasma TNF-α, leukocyte NF-κβ activity, plasma TAC and fecal serine protease are reported here.

2.2. Biological assays

2.2.1. Measurement of serum 25-hydroxy vitamin D

Blood samples in red-top tubes (no anticoagulant) were used for serum collection. Serum was separated by centrifugation at 200 ×g for 15 min. To assess pre-test vitamin D level of recruited participants, serum 25-hydroxy vitamin D level was assessed using a human serum 25-hydroxy vitamin D ELISA kit (Calbiotech, USA). Participants with serum levels higher than 50 ng/mL 25-hydroxy vitamin D were excluded from the study (data shown in ref. [17]).

2.2.2. Measurement of tumor necrosis factor-α

Blood samples (5 mL) were collected from participants before and after intervention. The plasma was separated by centrifugation at 200 ×g for 15 min. A human TNF-α ELISA kit (Zellbio Co., Germany) was used to determine plasma TNF-α level. The sensitivity of the test was 8 pg/mL plasma.

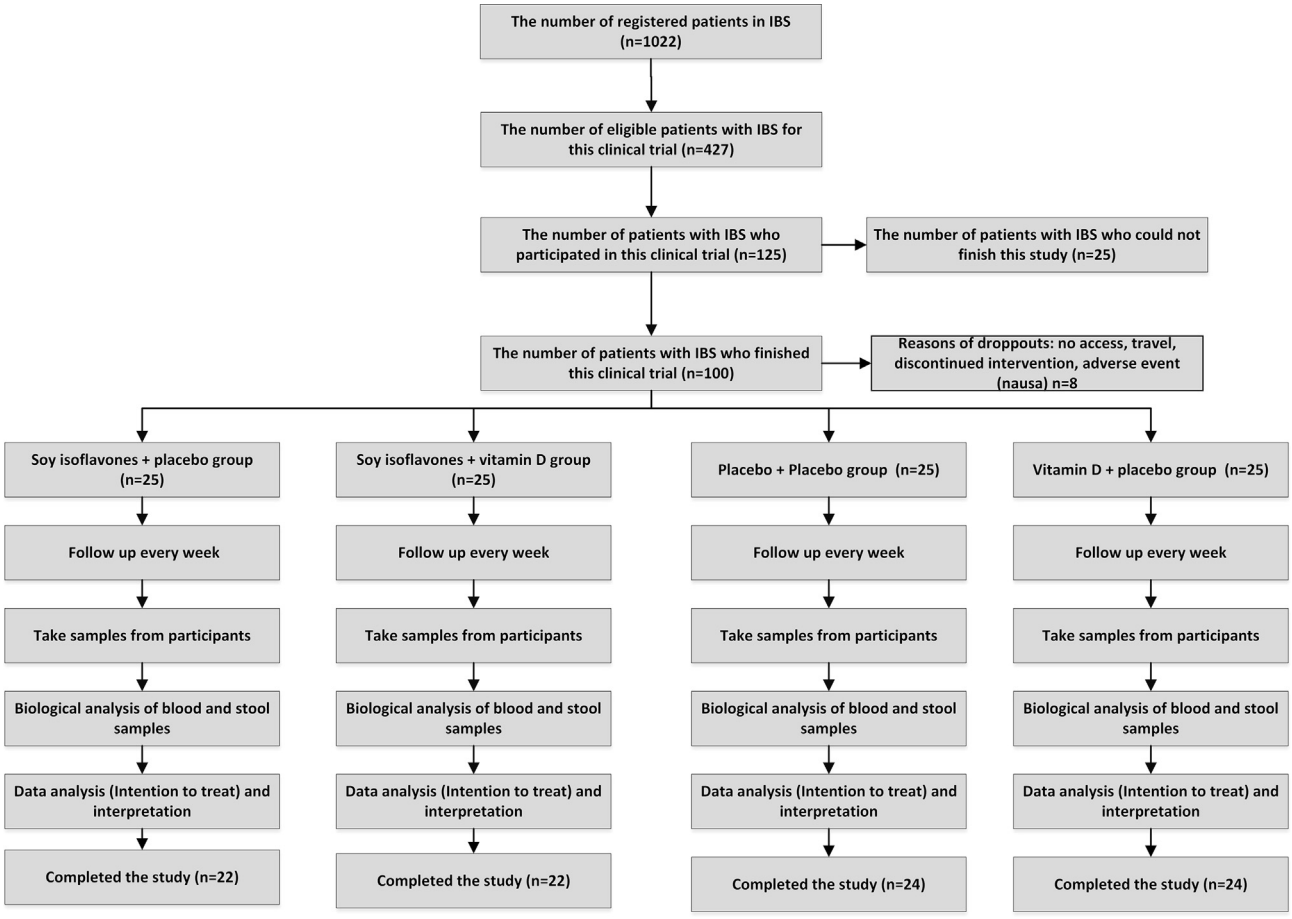

Fig. 1. Consort diagram of the study.

2.2.3. Measurement of nuclear factor-κβ

The buffy coat of the blood samples was separated by adding equal volumes of PBS and 2% FBS, followed by centrifugation at 200 ×g for 15 min. In the buffy coat containing the leukocyte band, a human NF-κβ ELISA kit (Zellbio Co., Germany) was used to measure leukocyte NF-κβ level. The sensitivity of the test was 10 pg/mL buffy coat.

2.2.4. Measurement of total antioxidant capacity

The plasma was separated by centrifugation at 200 ×g for 15 min. A human TAC calorimetric kit (Zellbio Co., Germany) was used to measure plasma TAC levels. The sensitivity of the test was 1 unit/mL plasma.

2.2.5. Measurement of fecal serine protease enzyme activity

Stool samples were collected in sterile containers at week 0 and week 6, and were stored at −80 °C until analysis. One gram of sample was dissolved in PBS and centrifuged at 200 ×g for 15 min. Serine protease activity in the supernatant liquid was determined using a Cusabio Biotech ELISA kit (China). The sensitivity of the test was 15.6 ng/mL.

Table 1
Baseline characteristics of study groups.

Characteristic[a]	Placebo + Placebo (n = 25)	Soy + placebo (n = 25)	Vitamin D + Placebo (n = 25)	Soy + vitamin D (n = 25)	P value[e]
Age (yr)[b]	39.37 (2.29)	42.54 (1.93)	40.69 (2.50)	38.65 (2.34)	0.605
Weight (kg)[b]	64.35 (2.09)	70.78 (1.79)	65.38 (2.18)	65.90 (2.44)	0.128
Height (cm)[b]	160.71 (1.09)	159.40 (0.92)	159.59 (1.00)	158.82 (1.08)	0.611
BMI (kg/m^2)[b]	25.35 (3.93)	28.08 (4.82)	26.04 (4.15)	26.49 (5.87)	0.225
Smoking[c]	3 (9.4%)	2 (5.7%)	1 (3.8%)	3 (9.4%)	0.849
Menopause[d]	9 (2.81%)	12 (34.3%)	8 (30.8%)	9 (28.1%)	0.939
IBS-C	22 (62.9)	19 (59.4)	21 (65.6)	12 (46.2)	0.374
IBS-D	4 (12.5)	5 (15.6)	3 (11.5)	3 (11.5)	
IBS-M	7 (20)	4 (12.5)	5 (15.6)	8 (30.8)	
IBS-U	2 (5.7)	4 (12.5)	2 (6.3)	3 (11.5)	

C: Constipation; D: Diarrhea; M: Mixed; U: Unsubtyped; Yr: year; Kg: kilogram; M: meter; cm: centimeter.
[a] - Mean (SEM).
[b] - Statistical significance test was done by ANOVA.
[c] - Statistical significance test was done by Fisher's exact test.
[d] - Statistical significance test was done by Pearson chi-square.
[e] P value < 0.05 was considered statistically significant.

2.3. Statistical analysis

The normal distribution of each variable was tested by Q-Q plot. Quantitative data are presented as mean ± SD. ANCOVA tests were used to analyze differences among groups after the intervention. The baseline level of each parameter was adjusted in the analysis. Statistical analysis was performed using SPSS software version 25.0 and graphs were prepared using by Sigma Plot version 14.0. $P < 0.05$ was considered to be statistically significant. Intention-to-treat analysis was used and any noncompliance was ignored after randomization.

3. Results

3.1. Plasma tumor necrosis factor-α level

There was a significant change among the groups after six weeks of supplementation. Post-hoc analysis after ANCOVA test showed a significant reduction of TNF-α in the soy + vitamin D and soy groups compared to placebo group ($p = 0.002$ and $p = 0.003$, respectively); however, there was no significant change in the vitamin D group compared to placebo group (Fig. 2).

3.2. Leukocyte nuclear factor-κβ level

NF-κβ levels were significantly lower in all three treatment groups compared with the placebo group ($p < 0.001$) (Fig. 3).

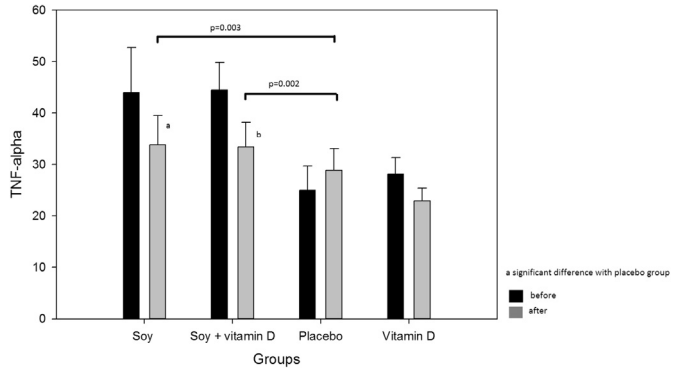

Fig. 2. Comparison of TNF-α level among treatment groups after six weeks. ANCOVA test and Bonferroni post hoc test. $P < 0.05$ significance level.

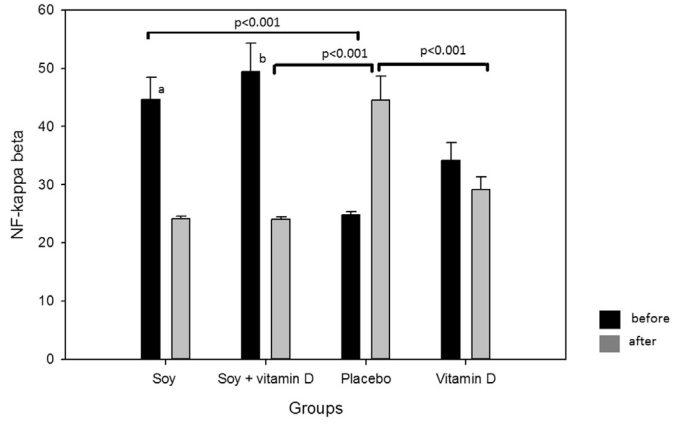

Fig. 3. Comparison of NF-κβ levels among treatment groups after six weeks. ANCOVA test and Bonferroni post hoc test. $P < 0.05$ significance level.

3.3. Plasma total antioxidant capacity

There was no significant difference after six weeks of supplementation among the treatment groups; however, there was a trend toward higher TAC levels in the soy and soy + vitamin D groups (Fig. 4).

3.4. Fecal serine protease enzyme activity

There was a significant difference among the groups after intervention. Post-hoc analysis after ANCOVA test revealed lower serine protease activity in the soy, soy + vitamin D and vitamin D groups compared to the placebo group ($p < 0.001$) (Fig. 5).

3.5. Adverse effects

No participants reported any side-effect during the study period.

4. Discussion

To our knowledge, this is the first randomized clinical trial to investigate the effect of soy and vitamin D alone and in combination on inflammatory markers, antioxidant status and gut permeability on patients with IBS in order to elucidate some molecular

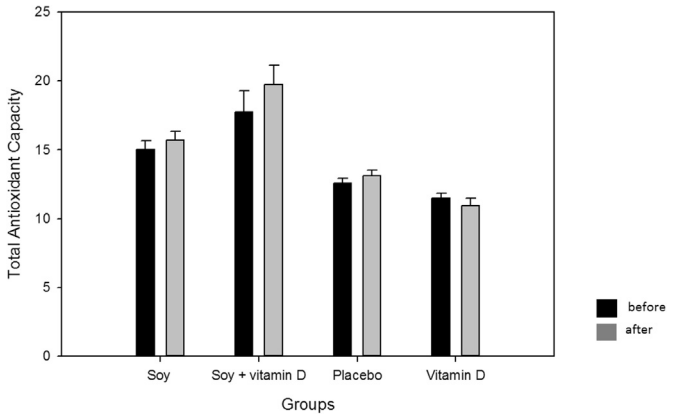

Fig. 4. Comparison of TAC levels among treatment groups after six weeks. ANCOVA test and Bonferroni post hoc test. $P < 0.05$ significance level.

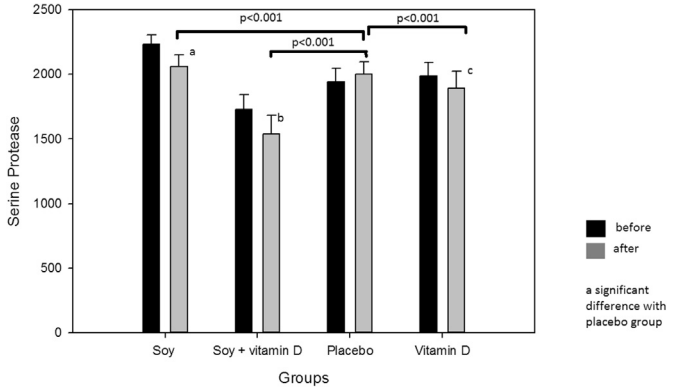

Fig. 5. Comparison of fecal serine protease activity among treatment groups after six weeks. ANCOVA test and Bonferroni post hoc test. $P < 0.05$ significance level.

mechanisms of action. The results of this study were consistent with our project hypothesis and showed that co-administration of soy isoflavones and vitamin D improved inflammatory markers and gut permeability; however, there was no significant change in plasma antioxidant status.

Few cell assay and animal experiments have been conducted to study the biological effects of supplementation in IBS. Suh et al. conducted a study on osteoblastic cells in vitro and found that genistein and daidzein inhibited TNF-α and other inflammatory markers (IL-6 and prostaglandin E_2). This study suggested that the anti-resorptive action of soy phytoestrogens is mediated by suppression of these inflammatory markers [19]. Another in vitro study on intestinal Caco-2 cells confirmed that soy phytoestrogens modulate IL-8 expression by suppressing TNF-α production, whereas other soy components such as saponins and peptides did not significantly affect the TNF-α and IL-8 pathways; thus, it appears that soy isoflavones may represent a promising prevention and treatment of IBS [20].

Despite several probable inflammatory markers for IBS etiology, genetic analysis of TNF-α alleles as one of major causes revealed that heterozygous TNF-α genotype (G/A) is more prevalent in patients with IBS (41%); thus, increased secretion of TNF-α in immunocompetent cells in intestinal mucosa is associated with higher probability of IBS, and significant suppression of TNF-α can be a reliable marker of treatment [4]. Since the molecular mechanism of intestinal permeability is associated with regulated activity of zonula occludens-1 proteins by TNF-α induced activity mediated by NF-κβ activation, intestinal permeability may be related to significant downregulation of TNF-α and NF-κβ in the same direction with lower serine protease activity and lower gut permeability [7].

There have been no clinical studies investigating the effects of soy isoflavones and vitamin D on TAC. One study investigating rat model has shown the positive effect of combined *Aloe vera* and *Matricaria recutita* as antioxidant sources on some antioxidant markers, such as myeloperoxidase and lipid peroxidation end products, in a rat model of IBS [10]. Similarly, Moussa et al. studied the positive effect of fermented soy germ as a source of phytoestrogen on suppression of fecal proteolytic activity and inhibited intestinal permeability [21]. Recently, Amani et al. observed a significant reduction of IL-17 and malondialdehyde and rise of plasma TAC in patients with IBS after supplementation with vitamin D. In that study, the dose of vitamin D was the same as in our experiment; however, the length of supplementation was longer [22]. Their different results may be due to long-term supplementation with vitamin D influencing pathophysiological molecular pathways such as interleukins and antioxidant markers, even if it is not co-administered with antioxidant ingredients such as isoflavones.

The strengths of this study include the high compliance level of participants, low rate of missing values and an appropriate study design to compare placebo effects in equal-size block randomization. The limitations of this study include the fact that gastrointestinal tract biopsies were not obtained, and that the outcome of treatment was not compared among the different IBS subtypes (i.e., IBS-diarrhea, IBS-constipation and IBS-mixed).

In conclusion, this clinical trial presents some biochemical evidence to indicate a significant impact of co-administration of soy isoflavones and vitamin D on inflammation level and gut permeability. Although supplementation with soy or vitamin D alone can improve the biomarkers significantly, the effect of co-administration is higher than those supplements separately. Future research could clarify the efficacy of these supplements by investigating a longer period or different doses, or could incorporate biopsy sampling or investigation of different TNF-α single-nucleotide polymorphisms (SNPs).

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MJ and AH designed the study; MJ, HP, and HV conducted the study; MJ analyzed the data, and prepared the primary draft of the manuscript; AH supervised the study and critically reviewed the manuscript.

There is no conflict of interest.

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