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Effect of Qing'e Formula on Circulating Sclerostin Levels in Patients with Postmenopausal Osteoporosis*

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Summary: Serum sclerostin is positively associated with serum 25 hydroxyvitamin D concentration. Our preliminary studies confirmed that Qing'e formula (QEF) could effectively increase serum 25 hydroxyvitamin D concentration in patients with postmenopausal osteoporosis (PMOP), but the effect of supplementation with QEF on serum sclerostin is unknown. This study investigated the effects of supplementation of QEF on serum sclerostin levels in patients with PMOP. Totally 120 outpatients and inpatients with PMOP treated in our hospital between January and October 2012 were randomly divided into QEF+calcium group, alfacalcidol+calcium group, and placebo+calcium group (n=40 each), with a follow-up period of 2 years. The serum levels of sclerostin, 25 hydroxyvitamin D, and bone turnover markers (β-CTX, N-MID and T-PINP) at baseline and at the 6th month, 1st year, 1.5th year, and 2nd year after treatment were measured. The results showed that the levels of circulating sclerostin were increased significantly at the 6th month after treatment in QEF+calcium group and alfacalcidol+calcium group as compared with placebo+calcium group (P<0.05), but there was no significant difference between the former two groups (P>0.05). The levels of β-CTX, N-MID and T-PINP in serum were decreased in both QEF+calcium group and alfacalcidol+calcium group at the 6th month after treatment, without significant difference between the two groups (P>0.05). But the levels were significantly lower than that in placebo+calcium group (P<0.05). These results suggest that the mechanism by which QEF modulates bone metabolism in patients with PMOP might be related with the effect of QEF in increasing sclerostin expression. Our findings provide a scientific rationale for using QEF as an effective drug to prevent bone loss in PMOP.
Key words: serum sclerostin; Qing'e formula; 25 hydroxyvitamin D; bone mineral density

Sclerostin, a glycoprotein produced by osteocytes, is evaluated as a potential clinical marker of bone turnover[1–4]. A recent report has identified a positive association between serum 25 hydroxyvitamin D and sclerostin levels in healthy women[5]. Sclerostin is known as an inhibitor of bone formation and several cross-sectional studies have reported inverse associations of serum sclerostin with a variety of biochemical markers of bone turnover[6–8]. Supplementation with alfacalcidol and calcium lowers other bone turnover marker levels by up to 10% and alters serum sclerostin levels[9].

The Qing'e formula (QEF), a Chinese herbal formula, has the efficacy of "nourishing livers and kidneys", "increasing energy and blood" and "regenerating bone marrows". Preliminary studies showed that QEF could effectively raise 25 hydroxyvitamin D and vitamin D receptor (VDR) mRNA expression in blood of ovariectomized rats and patients with postmenopausal osteoporosis (PMOP)[10, 11], but whether it alters serum sclerostin levels has not been elucidated.

To understand the mechanism by which QEF modulates osteocytes, it is necessary to elucidate the effect of QEF on serum sclerostin levels. Toward this end, we examined whether treatment with QEF for two years, when compared with calcium and placebo, altered serum sclerostin levels in PMOP.

1 MATERIALS AND METHODS

1.1 Patients and Grouping

Out of 177 recruited women, 57 did not meet the included criteria. Totally, 120 PMOP patients entered the study, with an average age of 53±6 years old. The subjects, including either inpatients or outpatients of the hospital, were randomly divided into three groups (n=40 each): QEF+calcium group, treated with combination of calcium and QEF; the alfacalcidol+calcium group, receiving treatment of combination of calcium and alfacalcidol; and the placebo+calcium group, given placebo and calcium treatments. The treatments lasted for a period of 2 years. The protocol was approved by Clinical Trial Ethic Committee at Tongji Medical College, Huazhong University of Science and Technology. Written informed

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consent was obtained from each participant before entering the study, which was conducted in accordance with the Declaration of Helsinki. Criteria for exclusion included following conditions: use of calcium or vitamin D supplements for 2 months prior to enrollment, affected by factors or medications influencing bone metabolism, kidney or liver diseases, and current cancer[12].

1.2 Preparation and Usage of QEF

The ingredients of experiment medicine QEF were listed as follows: Eucommia (fried with salt) 480 g, Psoralen (fried with salt) 240 g, walnut (fried) 150 g, Salvia 240 g and garlic 120 g. Garlic was steamed, dried, crushed into fine powder with Eucommia, Salvia and Psoralen and then sifted. Walnut was then mashed and grinded with the above powder before the mixture was further sifted and mixed. For every 100 g of the powder, 50–70 g of refining honey was added for producing the Qing'e pill[11, 13]. The experiment subjects were then allocated into 3 groups. QEF was administered three times daily with warm water plus Caltrate (Wyeth, USA) 600 mg daily in QEF+calcium group. In alfacalcidol+calcium group, alfacalcidol (Teva Pharmaceutical Industries Ltd., Israel) 0.25 μg twice daily plus Caltrate 600 mg daily were administrated. In placebo group, the patients were treated with Caltrate 600 mg daily to ensure patients' compliance and facilitate better experiment.

1.3 Collection of Clinical Data and Measurements of Bone Mineral Density (BMD)

The results of the physical examinations and surveys of all patients were recorded in detail before treatment. The dual-energy X-ray absorptiometry (Lunar Prodigy Advance; GE Healthcare, USA) was used to measure the BMD at lumbar spine (L2–L4) and femoral neck. The patients were placed in position according to manufacturer's instructions. The images were then captured and analyzed, and the results were reported with reference to the World Health Organization diagnostic criteria (T values and g/cm^2). The patient's variations of serial scan areas were adjusted to within the range of 5%. The precision of measurement in terms of coefficient of variation (CV) in the patient population (n=30) was 0.8% for L2–L4 and 1.1% for the femoral neck. One individual made all BMD measurements using the same equipment to minimize variation in the statistical analysis.

1.4 Measurements of Blood Bone Turnover Markers in Patients with PMOP

Blood samples were drawn from the cubital vein of patients at 7 am every day. The collected blood samples were then centrifuged for 15 min at 3000 r/min and 2–8°C, and then stored at −80°C. All blood samples were subsequently delivered to the same central biochemical laboratory for analysis of the levels of sclerostin, 25 hydroxyvitamin D, and bone turnover markers, such as the special sequence of β collagen (β-Crosslaps, β-CTX), N-terminal osteocalcin (N-MID), and total N-terminal propeptide of type Ⅰ procollagen (T-PINP). Human sclerostin ELISA kit (Yanhui biotechnology Co. Ltd, Shanghai, China) was purchased to detect sclerostin protein expression in serum. Serum 25 hydroxyvitamin D, β-CTX, N-MID, and T-PINP levels were measured using automated Roche electrochemilum inescence system (Roche Diagnostics GmbH, Germany). All the kits were performed in accordance with manufacturers' protocols. Intra- and inter-assay variations were <4%.

1.5 Measurements of Blood Sclerostin in Patients with PMOP

The sclerostin expression was assessed by using quantitative RT-PCR with FastStart Universal SYBR Green 1 PCR master mix (Rox, Roche, USA) in an ABI7300 real-time PCR system (Invitrogen, China). The sequences of the primers are shown as below: β-actin (F: GACCTGACAGACTACCTCAT, R: AGACAGCACTATGTTGGCAT); sclerostin (F: GCGTTCAAGAATGATGCCACG, R: CTGTACTCGGACACGTCTTTGG). The amount of target mRNA was normalized to that of β-actin mRNA. The reaction mixture contained 9 μL H_2O, 1.0 μL oligo (dT15), 2.0 μL goal RNA solution, 4.0 μL 5× buffer, 2.0 μL 10 mmol/L dNTPs, 1.0 μL RNA inhibitor, and 1.0 μL reverse transcriptase. The reaction conditions were as follows: 42°C for 30 min, 80°C for 5 min. The quantitative PCR reaction mixture contained 1.0 μL cDNA, 5.0 μL 10× buffer, 7.0 μL 25 mmol/L $MgCl_2$, 1.0 μL 10 mmol/L dNTPs, 0.8 μL forward primers (20 pmol/μL), 0.8 μL reverse primers (20 pmol/μL), 1.0 μL SYBR Green, and 0.5 μL Taq (5 U/μL). The cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C to 60°C for 15 s, and 60°C for 5 min. Next, the Ct values were calculated and analyzed by a formula: the sample relative value = $2^{-(\Delta Ct\beta\text{-actin}-\Delta Ct\ test\ sample)}$, $\Delta Ct = Ct_{negative\ control}-Ct_{sample}$, and $\Delta\Delta Ct = \Delta Ct_{\beta\text{-actin}}-\Delta Ct_{sample} = Ct_{sample}-Ct_{\beta\text{-actin}}$.

1.6 Statistical Analysis

All data are presented as $\bar{x}\pm s$. Statistical analyses were performed using the Kruskal-Wallis test or analysis of variance (ANOVA) followed by Student's t-test for multiple comparisons using the SPSS software (version 13.0, SPSS, Inc., USA). A P value of <0.05 was considered to be statistically significant.

2 RESULTS

2.1 General Conditions of Patients with PMOP in Three Groups

The baseline characteristics of the three groups were similar in the recruited population (table 1). Two subjects, one subject, and four subjects were drop-outs in QEF+calcium group, alfacalcidol+calcium group, and placebo+calcium group, respectively. The others were involved in the final analysis for two years. The incidence of all fractures was 7.9% in QEF+calcium group, 7.7% in alfacalcidol+calcium group, and 11.1% in placebo+calcium group, respectively, over 2 years. The incidence of fractures was not significantly different among the three groups (P>0.05).

2.2 Comparison of BMD among Three Groups

There was no statistically significant difference in BMD among the three groups at baseline. At the 6th month after treatment, BMD in QEF+calcium group was slightly increased in the lumbar spine and femoral neck as compared with that before the treatment (P>0.05). At the 12th–24th month after treatment, BMD in QEF+calcium group and alfacalcidol+calcium group was significantly higher than that in placebo+calcium group (P<0.05), but there was no significant difference between the former two groups. BMD in the placebo+calcium group was decreased significantly at the 12th month after treatment (P<0.05) (figs. 1 and 2).

Table 1 Baseline clinical characteristics and analysis of risk factors in three groups (n=40 each, $\bar{x}\pm s$)

Parameters	QEF+calcium group	Alfacalcidol+calcium group	Placebo+calcium group
Age (years)	58.26±3.45	58.54±3.63	58.42±3.56
Age at menopause (years)	53.76±2.65	53.94±2.74	53.42±2.36
Weight (kg)	59.64±5.69	60.04±6.01	58.98±5.67
Height (m)	1.62±0.05	1.64±0.11	1.61±0.04
BMI (kg/m^2)	23.63±2.52	24.06±2.23	23.54±2.46
Estradiol (pmol/mL)	37.15±12.56	38.51±12.77	38.18±13.06

BMI: body mass index. Student's t tests showed there were no significant differences in age, weight, height, BMI, and estradiol among the three groups.

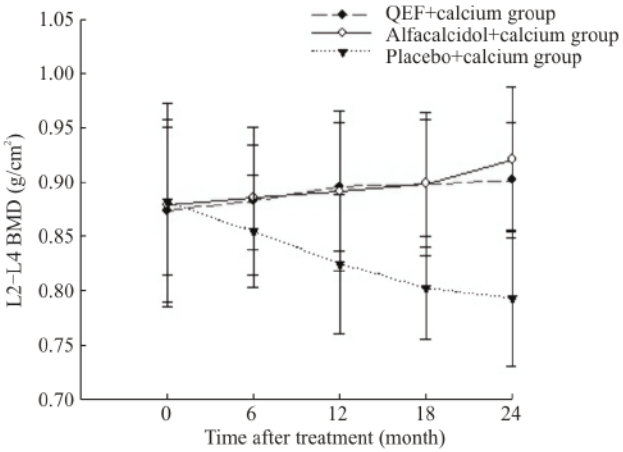

Fig. 1 BMD at L2–L4 at different time points in three groups

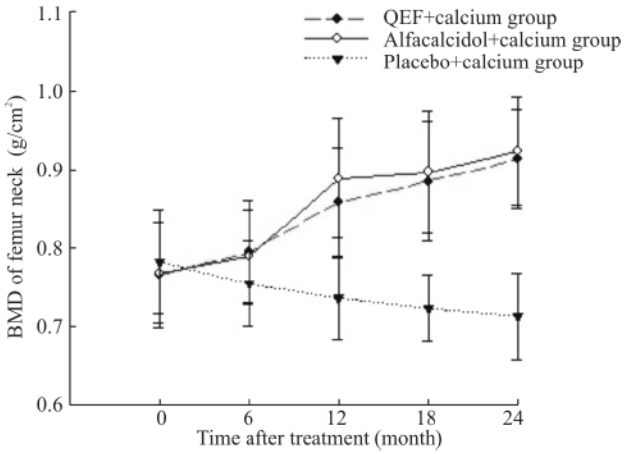

Fig. 2 BMD at femur neck at different time points in three groups

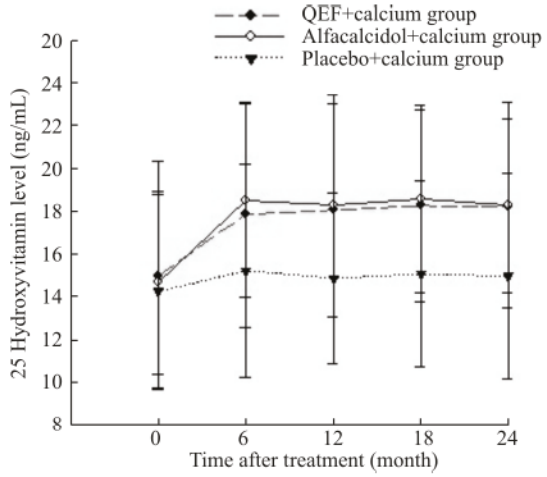

Fig. 3 Levels of 25 hydroxyvitamin D at different time points in three groups

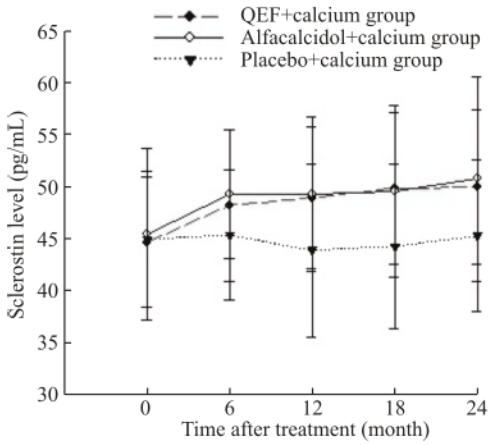

Fig. 4 Levels of sclerostin at different time points in three groups

2.3 Comparison of Levels of Sclerostin and 25 Hydroxyvitamin D in Three Groups

The levels of sclerostin and 25 hydroxyvitamin D in circulation system of the patients were not significantly different before treatment among the 3 groups. At the 6th to 24th months after treatment, the levels of sclerostin and 25 hydroxyvitamin D in serum were increased in both QEF+calcium group and alfacalcidol+calcium group, without significant difference between the two groups (P>0.05). But the levels in the former two groups were significantly higher than those in placebo+calcium group (P<0.05). The concentration of sclerostin and 25 hydroxyvitamin D was significantly increased at the 6th months, as compared with that at the 12th, 18th, and 24th months (P<0.05) (figs. 3 and 4).

2.4 Comparison of Levels of β-CTX, N-MID and T-PINP in Three Groups

The levels of β-CTX, N-MID and T-PINP in the blood of patients were not significantly different among the three groups at baseline. At the 6th to 24th months after treatment, the levels of β-CTX, N-MID and T-PINP in serum were decreased in both QEF+calcium group and alfacalcidol+calcium group, without significant difference between the two groups (P>0.05). But the levels were significantly lower than those in placebo+calcium group (P<0.05). The concentration of β-CTX, N-MID and T-PINP was significantly decreased at the 6th and 12th months, as compared with that at the18th and 24th months (P<0.05) (figs. 5, 6 and 7).

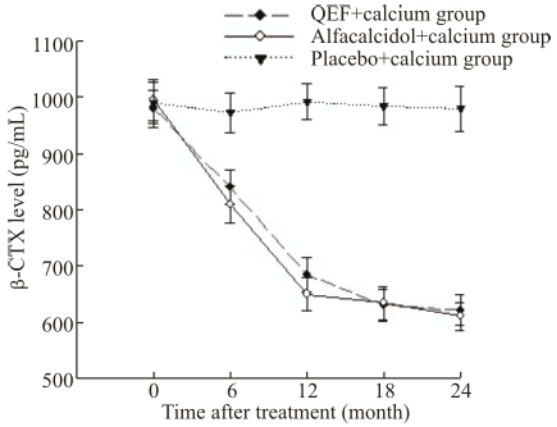

Fig. 5 Levels of β-CTX at different time points in three groups

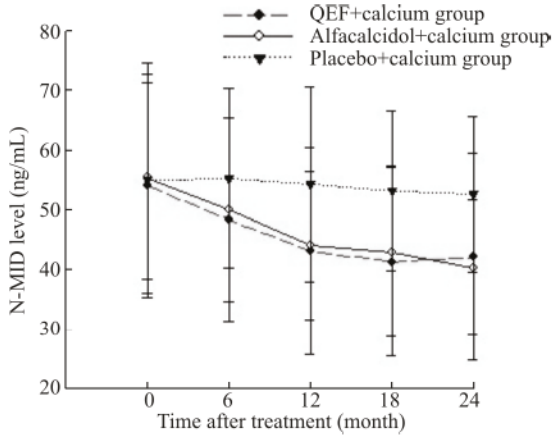

Fig. 6 Levels of N-MID at different time points in three groups

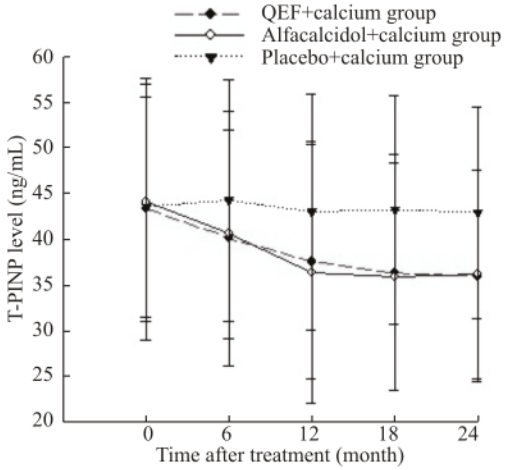

Fig. 7 Levels of T-PINP at different time points in three groups

2.5 Comparison of Sclerostin mRNA Expression among Three Groups

There was no statistically significant difference in sclerostin mRNA expression among the three groups at baseline. The expression of sclerostin mRNA was significantly increased in both QEF+calcium group and alfacalcidol+calcium group two years after treatment (P<0.05), without significant difference between the two groups (P>0.05). Sclerostin mRNA expression of both the QEF+calcium group and the alfacalcidol+calcium group was significantly higher than that of the placebo+calcium group two years after treatment (P<0.05; fig. 8).

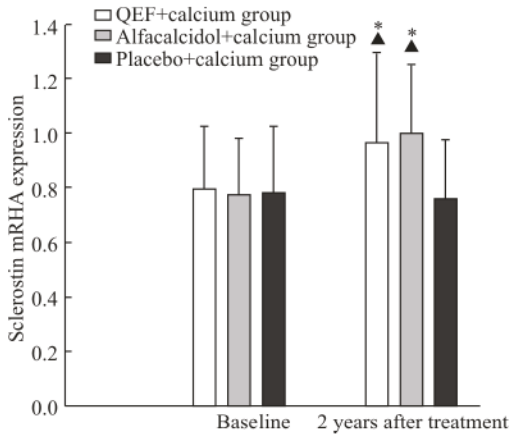

Fig. 8 Comparison of sclerostin mRNA expression in three groups
*P<0.05 *vs.* baseline, ▲P<0.05 *vs.* placebo group

3 DISCUSSION

Sclerostin is a secreted Wnt antagonist produced by osteocytes, and it can regulate bone mass by binding to low-density lipoprotein receptor-related protein-5 and -6 to inhibit the canonical Wnt/β-catenin signaling pathway. The biological importance of sclerostin in regulating bone mass in humans is highlighted by two genetic disorders associated with markedly increased bone mass, sclerosteosis and van Buchem's disease[14–17]. Sclerosteosis is caused by a mutation in the gene encoding sclerostin, SOST, which results in an improperly spliced SOST mRNA[14, 15], whereas van Buchem's disease is caused by a deletion of an enhancer element downstream of the SOST gene[16, 17]. These findings, combined with the demonstration that sclerostin-deficient mice had increased bone mass[18], have led to the development of antisclerostin-neutralizing antibodies as a novel anabolic treatment for osteoporosis[19]. According to the action of sclerostin on bone metabolism, a negative correlation between serum sclerostin and BMD would be expected as Mirza *et al*'s report[20]. However, a positive correlation between serum sclerostin and BMD was found in our another previous study[21] and similar results have been reported by Mödder *et al*[22] who found that BMD levels of the whole body and the lumbar spine were positively correlated with serum sclerostin levels in middle-aged women, both unadjusted and adjusted for age. Sheng *et al*[23] reported that serum sclerostin level was positively correlated with BMD levels in the whole body, the lumbar spine and the whole hip in postmenopausal women. For the counterintuitive phenomenon, a possible explanation has been proposed that sclerostin reflects number of osteocytes—assuming that this number is proportional to BMD and not the activity of individual cells or individual bone remodeling unit[4, 23]. This may partly explain why serum sclerostin was higher in PMOP patients treated by QEF/alfacalcidol and these patients had higher BMD in this study.

We demonstrated that treatment with QEF and calcium, as compared with placebo and calcium, increased serum 25 hydroxyvitamin D and sclerostin levels in the PMOP patients, and the effect was similar to that of

treatment with alfacalcidol and calcium. Our preliminary studies confirmed that QEF was effective in improving serum 25 hydroxyvitamin D concentration in ovariectomized osteoporosis rats and PMOP patients[10, 11]. These findings demonstrated that QEF and calcium supplements, similar to alfacalcidol and calcium supplements, increased 25 hydroxyvitamin D concentration in humans. Alfacalcidol and calcium supplementation may influence circulating sclerostin levels for following reasons. Supplementation with these nutrients decreases serum PTH levels[24], and PTH is a negative regulator of sclerostin expression[11, 25]. Hence the PTH decline should increase serum sclerostin levels. These findings also supported that QEF and calcium supplements, similar to alfacalcidol and calcium supplements, increased sclerostin levels. Even the long course of QEF treatment (2 years) in the present study was associated with marked increases in markers of bone formation.

β-CTX, N-MID and T-PINP are three types of bone turnover markers recommended by the International Osteoporosis Foundation. β-CTX is an organic component of bone matrix, of which 90% are type Ⅰ collagen synthesized in bones, but are also decomposed into degradation products that are released into the blood. β-CTX is a typical product of collagen degradation of type Ⅰ collagen. Examination of β-CTX can be used for monitoring the anti-resorptive treatment for osteoporosis or other bone diseases, and the efficacy can be reflected within several weeks. N-MID is produced by osteoblasts during bone synthesis. Both complete osteocalcin and large N-MID fragments are present in blood; however, the former is unstable and may split and degrade into the latter. The N-MID is regarded as a marker of bone synthesis, which can be used in conjunction with β-CTX for monitoring treatment progress of diseases such as osteoporosis. Concentration of total PINP in blood is one of the test items of bone markers, which can be used for monitoring treatment of osteoporosis in postmenopausal women clinically.

The results of this study show that QEF can reduce bone resorption through regulating sclerostin expression. This experiment used alfacalcidol as a reference drug for the control study, for it was reported to be stable and effective in a literature[24]. Data showed similar efficacy between QEF and alfacalcidol, as both are able to increase sclerostin expression in the body for reducing bone resorption and strengthening the quality and quantity of bones. The results suggest that the ancient recipe QEF has comparable efficacy in prevention and treatment of PMOP as appropriate supplement of alfacalcidol. These observations contribute to scattered findings reported in other data sets and, in themselves, do not indicate clinical utility of serum sclerostin measurements as indicators of bone turnover at this time.

It is a major limitation of our study that PTH value is vacant. Furthermore, it may be that circulating levels of the parameter we detected can not accurately reflect their levels in bone tissues. Further, carefully designed studies are required to verify our results and investigate the significance.

Conflict of Interest Statement

The authors declare that there is no conflict of interest with any financial organization or corporation or individual that can inappropriately influence this work.

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