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Vitamin D–enhanced eggs are protective of wintertime serum 25-hydroxyvitamin D in a randomized controlled trial of adults^{1,2}

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ABSTRACT

Background: Despite numerous animal studies that have illustrated the impact of additional vitamin D in the diet of hens on the resulting egg vitamin D content, the effect of the consumption of such eggs on vitamin D status of healthy individuals has not, to our knowledge, been tested.

Objective: We performed a randomized controlled trial (RCT) to investigate the effect of the consumption of vitamin D–enhanced eggs (produced by feeding hens at the maximum concentration of vitamin D₃ or serum 25-hydroxyvitamin D [25(OH)D₃] lawfully allowed in feed) on winter serum 25(OH)D in healthy adults.

Design: We conducted an 8-wk winter RCT in adults aged 45–70 y (*n* = 55) who were stratified into 3 groups and were requested to consume ≤2 eggs/wk (control group, in which status was expected to decline), 7 vitamin D₃–enhanced eggs/wk, or seven 25(OH)D₃–enhanced eggs/wk. Serum 25(OH)D was the primary outcome.

Results: Although there was no significant difference (*P* > 0.1; ANOVA) in the mean preintervention serum 25(OH)D in the 3 groups, it was ~7–8 nmol/L lower in the control group than in the 2 groups who consumed vitamin D–enhanced eggs. With the use of an ANCOVA, in which baseline 25(OH)D was accounted for, vitamin D₃–egg and 25(OH)D₃–egg groups were shown to have had significantly higher (*P* ≤ 0.005) postintervention serum 25(OH)D than in the control group. With the use of a within-group analysis, it was shown that, although serum 25(OH)D in the control group significantly decreased over winter (mean ± SD: −6.4 ± 6.7 nmol/L; *P* = 0.001), there was no change in the 2 groups who consumed vitamin D–enhanced eggs (*P* > 0.1 for both).

Conclusion: Weekly consumption of 7 vitamin D–enhanced eggs has an important impact on winter vitamin D status in adults. This trial was registered at clinicaltrials.gov as NCT02678364. *Am J Clin Nutr* 2016;104:629–37.

Keywords: bioaddition, RCT, vitamin D deficiency, vitamin D–enhanced eggs, 25(OH)D

INTRODUCTION

Data from the United States and Europe have suggested that 8% and 13% of the population, respectively (1, 2), have serum 25-hydroxyvitamin D [25(OH)D]⁸ concentrations <30 nmol/L,

which are reflective of vitamin D deficiency (3). Current dietary intakes of most populations, young and adult, on either side of the Atlantic are well short of dietary reference values for vitamin D (3–6), thereby contributing to the risk of vitamin D deficiency. The fortification of food with vitamin D has been suggested as a strategy with the potentially widest reach and impact in the population in terms of enhancing vitamin D intakes and minimizing the risk of vitamin D deficiency (6–9).

Although traditional fortification practices in which exogenous vitamin D is added to foods will continue to be an important approach for increasing the content of vitamin D (8), the use of the bioaddition approach to enhance the vitamin D content of foods (9) also merits serious attention not only because it may hold more consumer appeal in some cases but also because it may increase other metabolites of vitamin D that would boost the overall relative effectiveness of these foods in raising vitamin D status (7). For example, there have been several reports that have shown that the vitamin D₃ content of eggs can be substantially increased by the greater addition of vitamin D₃ to the feed of hens (10–16), albeit several of the studies (11, 12, 14, 15) used amounts for inclusion above the upper allowable amount for feeds in Europe [3000 IU/kg diet (17)]. The addition of commercially available 25(OH)D₃ to the diet of hens has also been shown to increase the 25(OH)D₃ content of eggs (13, 15, 16), albeit 2 studies used 25(OH)D₃ at amounts above the upper allowable amount [0.080 mg/kg diet (18)]. The use of a conversion factor of 5 [on the basis that supplemental 25(OH)D₃

¹ Research project funded the Irish Department of Agriculture, Food and Marine under its Food Institutional Research Measures.

² Supplemental Tables 1 and 2 are available from the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at <http://ajcn.nutrition.org>.

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⁸ Abbreviations used: CCVDNR, Cork Centre for Vitamin D and Nutrition Research; FFQ, food-frequency questionnaire; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MDI, mean daily intake; PTH, parathyroid hormone; RCT, randomized controlled trial; 25(OH)D, 25-hydroxyvitamin D.

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was 5 times more potent in raising the winter serum 25(OH)D of older men and women than was an equivalent amount of supplemental vitamin D₃ (19)] from our randomized controlled trial (RCT) to calculate the total vitamin D activity of vitamin D–enhanced eggs, showed that eggs from hens fed 25(OH)D₃ (3000 IU/kg diet) or an equivalent amount of vitamin D₃ had a total vitamin D activity of 5.1 and 3.8 μg/egg, respectively (16). However, this result assumes that the bioavailability of 25(OH)D₃ from supplements and food matrices is similar. In addition, other authors have reported lower conversion factors that ranged from 1 to 3 (20, 21), which would translate into eggs with an overall 0.7–3.6-μg lower total vitamin D activity because of the reduced impact of the 25(OH)D₃ content. Moreover, despite numerous studies on the impact of additional vitamin D in the diet of hens on the resulting egg vitamin D content (10–16), the effect of the consumption of such vitamin D–enhanced eggs on vitamin D status of healthy human subjects has not been tested in an RCT to our knowledge. A cross-sectional study of 564 school children (aged 9–12 y) in Spain showed that those who consumed <0.5 usual eggs/d had significantly lower serum 25(OH)D (by ~10 nmol/L) than children who consumed ≥0.5 eggs/d (22).

To date, studies on the sensory acceptability of vitamin D₃–enhanced eggs have been on eggs in which the hens received well above the European allowable upper amount (11, 14), and to our knowledge, 25(OH)D₃–enhanced eggs have not been tested. Therefore, the primary objective of the current work was to investigate the effect of the consumption of vitamin D₃– or 25(OH)D₃–enhanced eggs on winter serum 25(OH)D in adults in a 8-wk RCT. The secondary objective was to undertake a sensory evaluation of vitamin D–enhanced eggs to ascertain their consumer acceptability.

METHODS

Sensory analysis of vitamin D–enhanced eggs

Eggs from our previous hen feeding trial, whereby hens were fed one of 4 dietary treatments in which the vitamin D content or form differed (i.e., treatment 1: 1500 IU vitamin D₃/kg diet [this amount is one-half the upper allowable amount in Europe (17) and may be more reflective of that used in some commercial production, as evidenced by the wide variability in the vitamin D content of commercial eggs in Europe (13) as well as in the United States (23)]; treatment 2: 3000 IU vitamin D₃/kg diet; treatment 3, 1500 IU vitamin D₃ plus 1500 IU (37.5 μg) 25(OH)D₃ (HyD; DSM Nutritional Products Ltd.)/kg diet; and treatment 4: 3000 IU (75 μg) 25(OH)D₃/kg diet (16)), with the initial aim of identifying the dietary treatment that yielded eggs with the highest total vitamin D activity, were used for this associated sensory analysis. The total vitamin D activity [with the use of data derived from the National Food Institute, Technical University of Denmark, where the vitamin D₃ and 25(OH)D₃ contents of pooled egg-yolk samples for each treatment were measured with the use of liquid chromatography–tandem mass spectrometry (LC-MS/MS) (24) (pools were derived from *n* = 12–15 egg yolks/treatment)] was calculated as follows: vitamin D₃ + [25(OH)D₃ × 5]. The conversion factor of 5 that was applied to the 25(OH)D₃ content was based on data from our previous RCT (19), as mentioned previously, and this factor is

commonly used in several food-composition tables, including those in the United Kingdom (25–27). The mean ± SD total vitamin D activity in the pooled egg-yolk samples was 2.8 ± 0.5, 3.8 ± 0.3, 4.8 ± 0.8, and 5.1 ± 0.5 μg/egg for treatments 1–4, respectively (16). Eggs (both boiled and fried) were prepared and cooked as follows. In the case of boiled eggs, whole eggs were placed in a saucepan of cold water and boiled for 10 min on a conventional cooker hob. After cooking, eggs were cooled in cold water, the shells were removed, and each boiled egg was cut in half (longitudinally) before presentation to sensory panelists. In the case of fried eggs, eggs were cracked open and cooked (5 min in vegetable oil) within stainless-steel cooking rings placed in a frying pan to ensure the uniformity and consistency of fried eggs for sensory evaluation. Fried eggs were cut in half and reheated for 20 s in a microwave before presentation to sensory panelists.

A sensory evaluation of boiled and fried eggs was carried out in 2 separate sensory analysis sessions with the use of 22 and 18 consented, naive assessors, respectively, whereby 4 egg samples (one sample from each of treatments 1–4), which were identified with random 3-digit codes, were presented to each panelist in duplicate. The sample presentation order was randomized to prevent any flavor-carryover effects. The sensory analysis was undertaken in panel booths at a sensory laboratory in the School of Food and Nutritional Sciences, University College Cork, in accordance with International standards (28). Assessors were also provided with water and crackers to cleanse their pallets between samples.

Hedonic [appearance, liking of flavor, liking of texture, and overall acceptability (dislike or like; unacceptable or acceptable)] and intensity sensory-analysis descriptors [yolk color (yellow or orange), egg white (white or gray), sulfur flavor (none or extreme), sour (not or very), sweet (not or very), salty (not or very), odor (weak or strong), and off flavor (none or extreme)] were determined whereby assessors were asked to indicate their degree of liking on a 10-cm continuous line scale that ranged from zero to 10. Results for sensory analysis scores were measured in cm.

Subjects for RCT

A total of 55 apparently healthy, free-living adults, aged 45–70 y, were recruited into this 8-wk food-based vitamin D intervention trial. Subjects were recruited in the Cork area through the use of advertisements that were placed around University College Cork and across the location. We aimed to recruit approximately equal numbers of men and women. Inclusion criteria were consenting white men and women aged 45–70 y. Volunteers were excluded if they were unwilling to discontinue the consumption of vitamin D–containing supplements for 4 wk before the initiation of the study and throughout the study. Volunteers were also excluded if they planned to take a winter vacation (during the course of the 8-wk intervention) to a location where either the altitude or the latitude would be predicted to result in significant cutaneous vitamin D synthesis from solar radiation (e.g., a winter-sun coastal resort or a mountain ski resort) or if they used tanning facilities of any type. Additional reasons for exclusion were a severe medical illness, an allergy to egg products, being medically advised to limit egg intake in relation to managing hypercholesterolemia, hypercalcemia, known intestinal malabsorption syndrome, excessive alcohol use,

and the use of medications known to interfere with vitamin D metabolism. The study was approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals, University College Cork. All participants gave their written informed consent according to the Helsinki Declaration. This trial was registered at clinicaltrials.gov as NCT02678364.

Rationale and design of RCT

The study was a controlled, food-based intervention trial in which adults (aged 45–70 y) were randomly assigned to 3 groups. A control group, who habitually consumed only ≤ 2 eggs/wk and who agreed to do so for the duration of the RCT, and 2 vitamin D groups, who were habitual egg consumers and were willing to consume 7 eggs/wk, received either 7 vitamin D₃-enhanced eggs (vitamin D₃-egg group) or seven 25(OH)D₃-enhanced eggs [25(OH)D₃-egg group] a week for 8 wk. The rationale for the inclusion of a control group was to show that serum 25(OH)D concentrations decreased over the 8 wk of the winter RCT akin to a placebo group in our previous vitamin D RCTs (19, 29, 30). Our initial hen-feed trial showed that even the use of only one-half of the upper allowable amount of vitamin D in feeds led to eggs with a considerable total vitamin D activity (2.8 $\mu\text{g}/\text{egg}$) (16); thus, it was not feasible to supply 7 eggs/wk without having an impact on the vitamin D status of the control group. Therefore, we chose to limit the egg consumption to a maximum of 2 eggs/wk (participants were not required to consume these 2 eggs, but if they consumed eggs, they were asked to limit their consumption to 2 eggs/wk), and in terms of feasibility, we selected habitual low consumers of eggs for this control group to enhance compliance over the 8 wk. We felt that this was a more feasible approach than requesting participants who were habitual egg consumers and stratified to the control group to limit their egg intake to ≤ 2 eggs/wk over 8 wk, which could have been challenging. The 2 vitamin D groups were egg consumers who were willing to consume 7 eggs/wk. Seven and 1–2 eggs/wk (including eggs in dishes) were consistent with the amounts that are consumed in the highest and lowest tertiles, respectively, of adult egg consumers in Ireland on the basis of data from the National Adult Nutrition Survey (31). From a dietary guideline perspective, the general population can include ≤ 7 eggs/wk in their diets (32). The random assignment of subjects in the 2 vitamin D-enhanced-egg groups was centralized, computer generated, and accounted for sex.

Vitamin D intake from food sources in the control group was expected to be close to that in the general adult population aged 50–70 y (~ 5 $\mu\text{g}/\text{d}$) (5), whereas intakes in the 2 vitamin D-enhanced-egg groups were expected to be ~ 10 $\mu\text{g}/\text{d}$ on the basis of the additional vitamin D activity provided by the eggs. However, the ~ 10 $\mu\text{g}/\text{d}$ in the 25(OH)D₃-egg group was based on the assumption that the 25(OH)D in the eggs would behave in a similar manner to the synthetic 25(OH)D that was used by us in our previous RCT of older adults, which was shown to increase serum 25(OH)D more effectively (consumption of 1 $\mu\text{g}/\text{d}$ was equivalent to consumption of 5 μg vitamin D₃/d) (19), and for which some food-composition tables have assumed a factor of 5 (25–27). However, as mentioned previously, it may be that the bioavailability of 25(OH)D means that, at worst, it will only be equivalent to vitamin D₃ as has been suggested by some researchers (20). Thus, intakes of

vitamin D may have been only 6.5 $\mu\text{g}/\text{d}$ in the 25(OH)D₃-egg group.

Eggs for RCT

Both forms of vitamin D were approved by the European Commission expert group (Panel on Additives and Products or Substances used in Animal Feed) for inclusion in animal feed-stuffs (17, 18), and the vitamin D₃- and 25(OH)D₃-enhanced eggs for the RCT were supplied on a weekly basis by the School of Agricultural and Food Science, University College Dublin. The experimental vitamin D₃- and 25(OH)D₃-containing hen diets, each of which were produced at the beginning of the feeding trial in 100-kg batches, were based on a basic diet that contained crude protein (155 g/kg) and metabolizable energy (10.44 MJ/kg). The diets were also balanced for amino acid profiles and fatty acid contents and were provided in a layer mash form. Information on the composition of the hen diet used in the production of commercially available eggs was not known to us.

The eggs were placed in specifically purchased cardboard egg boxes labeled with the use-by date and subject IDs by staff who were not involved in the conduct of the RCT. The control (non-vitamin D-enhanced) eggs were purchased locally at a major retail store and were provided to all subjects in the control group for consistency. The vitamin D₃ and 25(OH)D₃ contents of the 3 types of eggs used in this trial [i.e., commercially available (control) eggs, vitamin D₃-enhanced eggs, and 25(OH)D₃-enhanced eggs] were assessed with the use of an in-house HPLC analysis within the Cork Centre for Vitamin D and Nutrition Research (CCVDNR), University College Cork.

Conduct of RCT

The study was carried out in Cork, Ireland (latitude: 51°N). All subjects were screened, recruited, and commenced the intervention study between 5 and 30 January 2015 and finished the study 8 wk later between 3 and 30 March 2015, during which the vitamin D status was expected to decline to a nadir (33). During the intervention phase, each participant was met by researchers on 2 sampling occasions at the human dietary studies unit at the CCVDNR at the baseline (week 0) and endpoint (week 8) of the study. At each visit, an overnight fasting blood sample was taken from each participant between 0830 and 1030 by a trained phlebotomist. Blood was collected by venipuncture into an evacuated tube with no additive and processed to serum, which was immediately stored at -80°C until required for analysis. Anthropometric measures, including height and weight, were taken as described previously (34). Habitual intake of vitamin D (and calcium) was estimated via a validated food-frequency questionnaire (FFQ) (35, 36) that was administered by a research nutritionist at the baseline. The FFQ, which had been tailored with the use of national food-consumption survey data obtained from Irish adults (aged 18–64 y) so as to identify foods that contribute 95% of vitamin D intake, has been shown to have a generally good level of agreement with a 14-d diet-history method and without a significant overall bias or proportional bias (36). Note that the FFQ was not designed to measure energy or other nutrient intakes and was designed to be administered by a trained researcher in association with validated methods of estimating



portion sizes (36). In addition, a health and lifestyle questionnaire, which assessed physical activity, general health, smoking status, and alcohol consumption, was completed at the baseline. Participants either collected their weekly and fortnightly allocation of fresh eggs at the human dietary studies unit, or in some cases when this was not feasible, the eggs were delivered to them. This regular contact also served to promote compliance and encourage the completion of the study protocol. The participants were asked to use the study-provided eggs in place of their normally purchased eggs for the duration of the study period. Subjects were requested to use the whole egg because egg white has a low vitamin D content (37). Subjects were provided with cooking suggestions and meal ideas for the incorporation of the vitamin D-enhanced whole eggs into their weekly diet. Compliance was assessed with the use of an egg diary. Although the control group were aware of their allocation by virtue of being limited to 2 eggs/wk, the allocation of the 2 vitamin D-enhanced-egg groups remained concealed from both participants and staff involved in the conduct of the RCT until the final analyses was complete. Biochemical outcome measures were reported by people who were masked to the allocation schemes of all subjects.

Laboratory analysis

HPLC analysis of RCT eggs

The separated yolks from 6 individual eggs/treatment were analyzed for vitamin D₃ and 25(OH)D with the use of an in-house HPLC method at the CCVDNR. The extraction procedure was adapted from Jakobsen et al. (38), and after semipreparative steps that have been described elsewhere (38, 39), vitamin D₃ and 25(OH)D were quantified with the use of an HPLC system (Shimadzu Corp.) consisting of 2 LC20ADXR pumps, an SIL-30AC autosampler, a CTO-20AC column oven, an SPD-30MA PDA detector, a FRC-10A fraction collector, and a CBM-20A system controller. Concentrations of vitamin D₃ and 25(OH)D₃ were calculated on the basis of an external calibration and adjusted for the percentage of recovery of the internal standard. In addition, because the eggs from our original hen-feeding trial were analyzed with the use of an LC-MS/MS method at the National Food Institute, Technical University of Denmark (24), the data were aligned with that method to ensure comparability. The National Food Institute laboratory has been accredited for vitamin D and 25(OH)D since 1994 and 2004, respectively. For the last 15 y, the laboratory has performed the analysis for vitamin D and 25(OH)D in food for the Danish and Norwegian food-composition tables.

Serum 25(OH)D

Concentrations of total 25(OH)D [i.e., 25(OH)D₂ plus 25(OH)D₃] in all serum samples were measured at the CCVDNR with the use of an LC-MS/MS method that has been described in detail elsewhere (40). The intra-assay CV of the method was <5% for all 25(OH)D metabolites, whereas the interassay CV was <6%. The CCVDNR is a participant in the Vitamin D Standardization Program (41) and is certified by the CDC's Vitamin D Standardization Certification Program (42). In addition, the quality and accuracy of the serum 25(OH)D analysis conducted with the use of LC-MS/MS in our laboratory is

monitored on an ongoing basis by participation in the Vitamin D External Quality Assessment Scheme (Charing Cross Hospital).

Serum intact parathyroid hormone

Serum parathyroid hormone (PTH) concentrations were measured at the CCVDNR in all serum samples with the use of an ELISA (intact PTH; MD Biosciences Inc.) Intra-assay and inter-assay CVs were 3.4% and 3.8%, respectively.

Serum total calcium and cholesterol

Total calcium, albumin, and cholesterol concentrations were measured at the CCVDNR in all serum samples with the use of a fully automated clinical analyzer (RX Monacol Randox Laboratories Ltd. Co.). Interassay CVs were 2.2%, 1.9%, and 2.2% for total calcium, albumin, and cholesterol, respectively. Serum calcium concentrations were adjusted for albumin concentrations.

Sample-size estimation

We wanted to be able to compare the endpoint (March) serum 25(OH)D concentration between the control group and the serum 25(OH)D concentrations in both of the vitamin D-enhanced-egg groups, which were predicted to have ~5 μg additional vitamin D/d. Our previous vitamin D RCTs in young and older adults (29, 30) have shown that there was a 12-nmol/L higher mean endpoint serum 25(OH)D concentration in the 5-μg vitamin D/d treatment groups than in the respective placebo groups. On the basis of the distribution of serum 25(OH)D data from our previous study of older adults, which was conducted at the same time of year (19), our power estimates, which were based on a 12-nmol/L difference in serum 25(OH)D between the control group and either of the vitamin D-enhanced-egg groups indicated a need for 17 volunteers/group ($n = 51$ in total) for 90% assurance at $\alpha = 0.5$. We did not power the study to detect differences between the 2 vitamin D-enhanced-egg groups because this was not the primary objective.

Statistical analysis

A statistical analysis of the sensory score data, which was conducted with the use of an ANOVA partial least-squares regression, was performed with Unscrambler software (version 10.3; CAMO ASA). The statistical analysis of the RCT data was conducted with the use of SPSS for Windows software (version 20.0; SPSS Inc.). Distributions of all variables were tested with the use of Kolmogorov-Smirnov tests. Descriptive statistics (means ± SDs or medians and IQRs when appropriate) were determined for all variables. A 1-factor ANOVA was used to compare the mean total vitamin D activity of 3 types of RCT eggs. Dietary calcium and serum PTH were not normally distributed and, thus, were log transformed to achieve near-normal distributions. Serum concentrations of 25(OH)D, albumin-corrected calcium, and total cholesterol as well as age, weight, height, BMI, and dietary vitamin D were normally distributed. Baseline characteristics of male and female subjects were compared with the use of unpaired Student's *t* tests. Baseline characteristics of subjects in the different intervention groups were compared with the use of a chi-square test (for the ratio of men to women) and a 1-factor ANOVA. An ANCOVA (with adjustment for the baseline variable being tested) was used to

test between intervention-group effects of treatment on weight as well as serum 25(OH)D, PTH, albumin-corrected calcium, and total cholesterol concentrations, and the Tukey's test was used for the post hoc analysis. Linear models of the response in a repeated-measures analysis for the differences in weight as well as of serum 25(OH)D, PTH, albumin-corrected calcium, and total cholesterol concentrations were also constructed. Main effects included dietary treatment and sex. Linear models also included 2-way interactions between main effects. Paired *t* tests were used to test changes within intervention groups from preintervention to postintervention concentrations of serum 25(OH)D, PTH, albumin-corrected calcium, and total cholesterol concentrations. Subjects included in the analyses and those who were not included (i.e., dropouts) were compared on the basis of selected characteristics with the use of 2-sample *t* tests (continuous variables) and chi-square tests (categorical variables). $P < 0.05$ was considered significant.

An intention-to-treat analysis of the serum 25(OH)D data as the primary outcome measure was also performed to account for the 4 subjects who dropped out. The 4 missing postintervention values for serum 25(OH)D were imputed with the use of the multiple-imputation function in the SPSS program ($k = 5$) as suggested by Armijo-Olivo et al. (43). This analysis was viewed as confirmatory of that in which the 4 dropouts were excluded.

RESULTS

Sensory evaluation of vitamin D-enhanced eggs

An ANOVA partial least-squares regression analysis of the sensory evaluation data on vitamin D-enhanced eggs from our initial hen trial (16) showed that there were no significant ($P > 0.05$) differences in hedonic or intensity sensory scores between vitamin D₃ and 25(OH)D₃ eggs (treatments 2 and 4, respectively) whether as boiled eggs (**Supplemental Table 1**) or as fried eggs (**Supplemental Table 2**). The consumer acceptability of eggs from hens that were fed treatments 2 and 4 did not differ from each other or from the other 2 treatments (i.e., treatments 1 and 3) (Supplemental Tables 1 and 2).

Vitamin D activity of eggs for RCT

The HPLC analysis of the 3 types of eggs used in the current RCT ($n = 6$ eggs/group) showed that the means \pm SDs of vitamin D₃, 25(OH)D₃, and total vitamin D activity (i.e., vitamin D₃ + [25(OH)D₃ \times 5]) for vitamin D₃ eggs were 1.04 ± 0.54 , 0.50 ± 0.10 , and 3.54 ± 1.04 $\mu\text{g}/\text{egg}$, respectively. The means \pm SDs of vitamin D₃, 25(OH)D₃, and total vitamin D activity for 25(OH)D₃ eggs were 0.14 ± 0.08 , 0.88 ± 0.26 , and 4.54 ± 1.38 $\mu\text{g}/\text{egg}$, respectively. The means \pm SDs of vitamin D₃; 25(OH)D₃; and total vitamin D activity for the commercially available (control) eggs were 0.63 ± 0.22 , 0.56 ± 0.22 , and 3.43 ± 1.31 $\mu\text{g}/\text{egg}$, respectively. There was no significant difference in the mean total vitamin D activity across the 3 types of eggs ($P = 0.13$; ANOVA).

Baseline characteristics of subjects in the RCT

Of 55 subjects who were recruited onto the study, 51 subjects completed the intervention. The progress of these subjects through the trial is shown in **Figure 1**. Baseline characteristics of

subjects who entered the intervention are shown in **Table 1**. Although women were, on average, lighter and smaller than men (both $P < 0.0001$) and had lower BMI ($P < 0.05$) and habitual intake of calcium ($P < 0.05$), there was no difference ($P > 0.2$ for all) in the mean age, habitual intakes of vitamin D, serum 25(OH)D, PTH, albumin-corrected calcium, or cholesterol concentrations between men and women at baseline (data not shown).

Effects of intervention with vitamin D-enhanced eggs

There was no difference ($P > 0.3$) in the mean age, weight, height, or BMI at baseline in the 3 treatment groups [control, vitamin D₃-egg, and 25(OH)D₃-egg groups] (data not shown). Similarly, there was no significant difference ($P > 0.9$) in the proportion of men to women in treatment groups (Table 1). Habitual dietary vitamin D and calcium intakes are shown in Table 1, and these intakes were similar in the 3 treatment groups ($P > 0.1$).

There were no adverse events reported during the study. Of 4 dropouts, one subject was from the control group, one subject was from the vitamin D₃-egg group, and 2 subjects were from the 25(OH)D₃-egg group. A dropout during the intervention phase was for reasons of a loss of interest or a back injury, and in no instance was a dropout related to the intervention. The 51 subjects who completed the study did not differ from the 4 subjects who dropped out with respect to age, sex, weight, height, BMI, vitamin D intake, calcium intake, or baseline serum 25(OH)D, PTH, serum albumin-adjusted serum calcium, or total cholesterol concentrations ($P > 0.16$).

There was good adherence with the egg-consumption protocols on the basis of the egg-diary entries [median (IQR) compliance was 98.2% (93.7–100%)], and compliance was similar in the treatment groups ($P = 0.6$).

Data on egg compliance and measured vitamin D and 25(OH)D contents of RCT eggs as well as habitual vitamin D intake data from the FFQ were used to estimate daily total vitamin D intakes throughout the study period. With the use of a factor of 5, mean \pm SD total vitamin D intakes were 9.5 ± 3.5 , 10.4 ± 4.0 , and 6.6 ± 3.9 $\mu\text{g}/\text{d}$ in the vitamin D₃-egg, 25(OH)D₃-egg, and control groups, respectively.

There was no difference in mean serum total cholesterol or body weight at baseline ($P > 0.4$), and there was no significant change in weight ($P = 0.39$) or serum total cholesterol ($P = 0.46$) from preintervention to postintervention in the 3 treatment groups (Table 1).

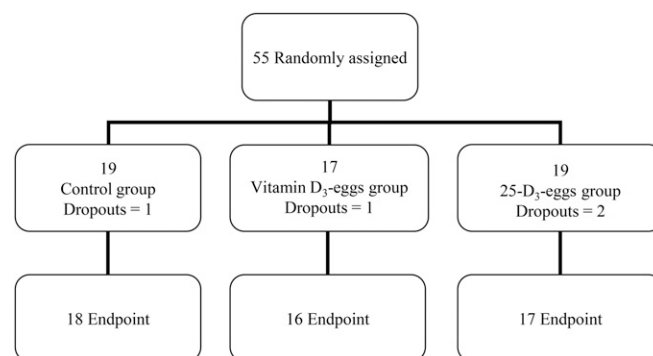


FIGURE 1 Flow of subjects through the study. 25-D₃, 25-hydroxyvitamin D₃.

TABLE 1

Selected baseline characteristics of subjects who entered the intervention study, weight, and serum total cholesterol, 25 (OH)D, and PTH concentrations in treatment groups at the baseline and endpoint of the 8-wk intervention in apparently healthy adults¹

	Treatment group			<i>P</i>
	Control	Vitamin D ₃ eggs	25(OH)D ₃ eggs	
Baseline, <i>n</i>	19	17	19	—
Sex, M:F, <i>n</i>	9:10	8:9	10:9	0.930
Age, y	55.8 ± 7.5 ²	56.2 ± 5.5	53.8 ± 5.8	0.498
Height, m	1.70 ± 0.12	1.70 ± 0.09	1.71 ± 0.09	0.807
BMI, kg/m ²	25.8 ± 3.5	26.5 ± 5.0	24.4 ± 3.7	0.300
Habitual dietary vitamin D, μg/d	6.0 ± 4.0	6.7 ± 3.5	6.9 ± 4.0	0.796
Habitual dietary calcium, mg/d	842 (670, 1014) ³	961 (764, 1739)	762 (666, 1257)	0.115
Endpoint, <i>n</i>	18	16	17	—
Weight, ⁴ kg				
Preintervention	75.1 ± 12.7	78.3 ± 20.1	73.4 ± 16.1	0.654
Postintervention	72.8 ± 15.6	78.2 ± 19.9	72.3 ± 15.6	0.387
Serum total cholesterol, ⁴ mmol/L				
Preintervention	5.2 ± 0.8	5.3 ± 0.9	5.5 ± 0.8	0.435
Postintervention	5.0 ± 0.8	5.2 ± 0.9	5.4 ± 0.6	0.463
Serum 25(OH)D, ⁴ nmol/L				
Preintervention	41.2 ± 14.1	48.2 ± 18.9	49.4 ± 15.8	0.102
Postintervention	34.8 ± 11.4 ^{a*}	50.4 ± 21.4 ^b	49.2 ± 16.5 ^b	<0.0001
Serum PTH, ⁴ ng/mL				
Preintervention	41.5 (35.0, 62.3)	48.9 (40.4, 53.9)	43.0 (39.6, 59.5)	0.991
Postintervention	47.0 (39.0, 68.4)	47.7 (36.8, 58.5)	44.9 (37.4, 58.6)	0.606

¹All preintervention (baseline) blood samples were taken between 5 and 30 January 2015. All postintervention (endpoint) blood samples were taken between 3 and 30 March 2015. *P* values for baseline comparisons by intervention group were determined with the use of a 1-factor ANOVA. *P* values for endpoint comparisons by intervention group were determined with the use of an ANCOVA (with adjustment for the baseline variable being tested) followed by Tukey's test. Different superscript letters represent significant (*P* ≤ 0.005) differences in group means. *Value was significantly different from the baseline value within the group, *P* = 0.001 (paired *t* test). PTH, parathyroid hormone; 25(OH)D, 25-hydroxyvitamin D; 25(OH)D₃, supplemental 25-hydroxyvitamin D₃.

²Mean ± SD (all such values).

³Median; IQR in parentheses (all such values for nonnormally distributed variables).

⁴Repeated-measures ANOVA was used to test the treatment × time interaction, and the same trend was observed for weight (*P* = 0.38), serum total cholesterol (*P* = 0.86), serum 25(OH)D (*P* = 0.0001), and PTH (*P* = 0.6). There was no significant (*P* > 0.2 in all cases) interaction with sex.

Effects of vitamin D–egg treatment on serum 25(OH)D and PTH at the baseline and endpoint are shown in Table 1. Although the preintervention serum 25(OH)D concentration of the control group was lower than that of the other 2 groups, it was not significantly so (Table 1). A repeated-measures ANOVA showed a significant treatment × time effect (*P* = 0.0001). The within-group analysis showed that, although the control group had a significant decrease in serum 25(OH)D over the 8-wk winter intervention period (mean ± SD: 6.4 ± 6.7 nmol/L; *P* = 0.001), there was no significant change in serum 25(OH)D over the same 8-wk period in the vitamin D₃-egg and 25(OH)D₃-egg groups (*P* > 0.1 for both). The ANCOVA also showed there was a significant (*P* = 0.001) effect of treatment on postintervention serum 25(OH)D concentrations whereby, with adjustment for preintervention serum 25(OH)D concentrations, the vitamin D₃-egg group and 25(OH)D₃-egg group had significantly higher (*P* ≤ 0.005) postintervention serum 25(OH)D concentrations compared with those in the control group. There was no significant (*P* > 0.3) difference in postintervention serum 25(OH)D concentrations between the vitamin D₃-egg and 25(OH)D₃-egg groups (Table 1).

The percentage (*n*/total group *n*) of subjects in each of the 3 groups who had postintervention serum 25(OH)D concentrations <25 nmol/L [which is the European Union threshold of vitamin D deficiency (44)] was 22.2% (4/18), 0% (0/16), and 0% (0/17) for the control, vitamin D₃-egg, and 25(OH)D₃-egg groups, respectively (*P* = 0.019). The percentage (*n*) of subjects in each of the 3 groups who had postintervention serum 25(OH)D concentrations <30 nmol/L [which is the US threshold of vitamin D deficiency (3)] was 38.9% (7/18), 18.8% (3/16), and 5.9% (1/17) for the control, vitamin D₃-egg, and 25(OH)D₃-egg groups, respectively (*P* = 0.057).

There was no significant difference in preintervention or postintervention serum PTH concentrations in the 3 treatment groups (Table 1). A repeated-measures ANOVA confirmed a lack of a significant treatment × time effect (*P* > 0.6). The within-group analysis showed that although there was a trend toward a significant increase in serum PTH (*P* = 0.050) in the control group over the 8-wk winter intervention period, there was no significant change in serum PTH over the same 8-wk period in the vitamin D₃-egg and 25(OH)D₃-egg groups (*P* > 0.7 for both). There was no significant interaction between treatment and sex in any of the serum 25(OH)D or PTH statistical analyses (*P* > 0.18 for both).



An intention-to-treat analysis of postintervention serum 25(OH)D (with the use of inputted values for the 4 subjects who dropped out during the study) showed similar findings ($P \leq 0.0001$) with a significant decrease in the control group ($P \leq 0.002$) but no significant difference in either of the 2 vitamin D-enhanced-egg treatment groups ($P > 0.17$) (data not shown).

DISCUSSION

Summary of main findings

Despite numerous vitamin D and 25(OH)D₃ hen-feeding studies over the past 15 y, which collectively have clearly shown the viability of increasing the vitamin D or 25(OH)D content of eggs (10–16), the current study, to our knowledge, is the first to investigate the effect of such vitamin D-enhanced eggs on vitamin D status in humans. The current RCT showed that the consumption of 7 vitamin D- or 25(OH)D₃-enhanced eggs/wk, both of which were shown to possess consumer acceptability and had no effect on weight or serum total cholesterol, maintained serum 25(OH)D concentrations in healthy adults and protected against its decline during the 8 wk of winter. Such a decline in serum 25(OH)D concentrations between January and March was evident in the control group, who consumed ≤ 2 commercially available eggs/wk. This decline in serum 25(OH)D is in line with that reported in the placebo groups in our previous RCTs of adults over a similar time frame during the winter (19, 45). Also note that the vitamin D-enhanced eggs, which exhibited these vitamin D-status protective effects, were achieved by the addition of either vitamin D₃ (3000-IU/kg diet) or 25(OH)D₃ (0.075-mg/kg diet) to the hens' feeds at amounts that are allowable by European Council directives (17, 18), whereas the majority of previous studies used amounts that exceeded these maximum allowable contents (10–12, 14, 15).

The consumption of the enhanced eggs as part of the participants' usual diets led to a mean daily intake (MDI) of $\sim 10 \mu\text{g}$ vitamin D/d, which, in turn, resulted in postintervention mean serum 25(OH)D concentrations $> 40 \text{ nmol/L}$. These results are consistent with the Institute of Medicine's estimated average requirement of $10 \mu\text{g/d}$ (3) even at a time of the year during which the vitamin D status would be expected to decline to a nadir (33). The vitamin D MDI of $\sim 10 \mu\text{g/d}$ in both vitamin D-enhanced-egg groups was also sufficient to prevent, or dramatically diminish, the prevalence of vitamin D deficiency depending on the selection of serum 25(OH)D threshold to define deficiency. For example, none of the participants in either of the 2 vitamin D-enhanced-egg groups had postintervention serum 25(OH)D concentrations $< 25 \text{ nmol/L}$ [i.e., the United Kingdom and European Union definition of vitamin D deficiency (44, 46)], whereas 6–19% of participants had postintervention serum 25(OH)D concentrations $< 30 \text{ nmol/L}$ [the US definition of vitamin D deficiency (3)]. In contrast, the prevalences of serum 25(OH)D concentrations < 25 and $< 30 \text{ nmol/L}$ were 22% and 39%, respectively, in the control group. However, because baseline serum 25(OH)D concentrations were lower, even if not significantly so, in this group than in the 2 vitamin D-enhanced-egg groups, it may have led to the slightly higher endpoint prevalence of vitamin D deficiency compared with that in the

placebo groups in our previous vitamin D RCTs [$\sim 33\%$, on average, with serum 25(OH)D concentrations $< 30 \text{ nmol/L}$ (29, 30, 45)]. The increase in serum PTH in the control group failed to reach significance, which may have been related to the magnitude of the drop in serum 25(OH)D (6.4 nmol/L). There was no change in serum PTH in the 2 vitamin D-enhanced-egg groups, which might have been expected because of the lack of a change in serum 25(OH)D over the 8 wk.

Consumer acceptability of vitamin D-enhanced eggs

It was also important that the 25(OH)D₃- or vitamin D₃-derived eggs had equal consumer acceptability profiles on the basis of the current sensory evaluation because any alteration to a sensory perception of a food that may be deemed negative by the consumer will affect their decision to consume the product. Two previous studies have shown that eggs produced from hens that were given additional vitamin D₃ in their feeds had similar sensory profiles as control eggs (11, 14), but the studies tested amounts of addition well above the European allowable upper amount, and neither study included eggs from hens that were fed 25(OH)D₃.

Role of 25(OH)D in the vitamin D-enhanced eggs

The current study did not set out specifically to answer the question of whether the relative potency of 25(OH)D to vitamin D₃ is 5 or some other factor as has been assessed in previous human and pig studies (19–21, 39, 47); instead, we wished to test the impact of vitamin D-enhanced eggs on human vitamin D status. In our study, it was assumed that 25(OH)D has a higher effectiveness in terms of raising serum 25(OH)D than vitamin D₃ does. For example, the calculation of total vitamin D activity of the eggs applied a conversion factor of 5 for 25(OH)D, as reported by us previously (19), and this factor has been used in several food-composition tables (25–27). The use of these total vitamin D-activity estimates meant that the vitamin D₃-egg and 25(OH)D₃-egg groups received an additional 3.5 and 4.5 μg vitamin D/d, respectively, which, together with the habitual dietary vitamin D, yielded an MDI $\sim 10 \mu\text{g/d}$. Of note, the current USDA National Nutrient Database for Standard Reference suggests that the vitamin D content of 100 g raw egg is 2 μg (48), an estimate that does not include 25(OH)D. Accordingly, estimates of vitamin D intake from the NHANES in the United States did not account for the contribution of 25(OH)D (4). Taylor et al. (49) recently performed some modeling to include food-derived 25(OH)D in intake estimates for US adults, the effect of which would be even more pronounced should vitamin D-enhanced eggs become more widespread in the United States. For example, the total vitamin D content of the eggs used in the analysis by Taylor et al. (49) at 5.7 $\mu\text{g}/100 \text{ g}$ whole egg, and which was very similar to that in the commercial eggs and vitamin D₃-enhanced eggs that were used in this study (5.7 and 6.0 $\mu\text{g}/100 \text{ g}$ whole egg, respectively), could be increased to 7.7 $\mu\text{g}/100 \text{ g}$ whole egg if 25(OH)D₃ eggs were used. In the United States, the Food and Drug Administration designated 25(OH)D as having a generally recognized as safe status for inclusion in chicken feeds (0.069-mg/kg diet) (50).

Strengths and limitations

This RCT of the impact of vitamin D-enriched eggs on vitamin D status in adults, which is the first such study to our knowledge, was underpinned by hen-feeding studies to specifically produce vitamin D₃- or 25(OH)D₃-enhanced eggs and also by sensory trials to ensure the consumer acceptability of the eggs. The purposeful selection of habitual low consumers of eggs (≤ 2 eggs/wk) in the control group for the pragmatic reasons of feasibility and to enhance compliance to limited egg consumption may have led to some degree of allocation bias. The selection may have contributed to the lower baseline serum 25(OH)D than that in the vitamin D-enhanced-egg groups, although it was not significantly so. In turn, the lower baseline 25(OH)D might have contributed to a lower postintervention serum 25(OH)D in the control group and could explain the measured difference of 14.4–15.6 nmol/L between control and vitamin D-enhanced-egg groups compared with the more-predicted 12-nmol/L difference. However, the statistical analysis took baseline serum 25(OH)D concentrations into account, and moreover, the repeated-measures ANOVA and post hoc tests showed that there was no change in serum 25(OH)D concentrations from preintervention to postintervention in either vitamin D-enhanced-egg group despite a significant decrease in the control group. The current study did not include a wider dietary assessment tool and, thus, did not assess the effect of the consumption of 7 vitamin D-enhanced eggs/wk on fat or energy intake; however, body weight and serum cholesterol were unaffected over the 8 wk of the RCT. Finally, although the inclusion of vitamin D₃ or 25(OH)D₃ in hen feeds led to the production of vitamin D-enhanced eggs with vitamin D-status protective effects in the current study, it should be stressed that the hens would have received commercial diets that contained vitamin D₃ before the commencement of the experimental diets. Therefore, it is possible that hens that are placed on diet with 25(OH)D₃ only (and no vitamin D₃) from a very-early age may produce eggs with an altered total vitamin D activity.

In conclusion, the weekly consumption of 7 vitamin D-enhanced eggs, which are produced by hens that are provided with feed containing vitamin D [either as vitamin D₃ or 25(OH)D₃] at the allowable maximum content has an important impact on winter vitamin D status in adults.

The authors' responsibilities were as follows— JK, AK, JO, KMS, and KDC: were involved in the conception of the study; AH, KMS, and KDC: contributed to the study design; AH, SD, MO, JT-D, SH, KMS, and KDC: contributed to the execution of the study; SD, AK, and JO: produced the vitamin D-enhanced eggs; AH, JJ, and KG: conducted the vitamin D and 25(OH)D analyses of the eggs; MO and JK: conducted the sensory analysis of the eggs; AH and JT-D: contributed to the sample analyses; and all authors: contributed to the data analysis and writing of the manuscript. None of the authors reported a conflict of interest related to the study.

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