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Food fortification and biofortification as potential strategies for prevention of vitamin D deficiency

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Running title: 25-hydroxyvitamin D_3 fortified foods

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Abstract

Hypovitaminosis D is widespread throughout the world. The cutaneous production of vitamin D through sunlight can be limited by several factors (*e.g*. skin pigmentation, sunscreen usage and, increasingly, indoor lifestyle). Thus, diet has become an important strategy to increase vitamin D intake and status. However, there are a limited number of foods (*e.g.* eggs, oily fish and wild mushroom) naturally enriched with vitamin D, and concentrations can vary significantly between and within species. Therefore, the need for vitamin D fortified foods (including via direct fortification and biofortification) to support adequacy of vitamin D status [blood 25-hydroxivitamin D (25(OH) D)] is a corollary of several limitations to synthesise vitamin D from sunlight. Ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3) can be found in some mushrooms and animal-derived foods, respectively. Evidence has shown vitamin D_3 is more effective than vitamin D_2 at raising 25(OH) D blood concentrations. The vitamin D metabolite, 25(OH) D_3, is present in animal-derived foods (*e.g*. meat, eggs and fish), and several intervention trials have shown 25(OH) D_3 to be more effective at raising blood 25(OH) D concentrations than vitamin D_3. In addition, 25(OH) D_3 supplements may prove to be preferable to vitamin D_3 for patients with certain clinical conditions. However, there is limited evidence on the effect of 25(OH) D_3 fortified foods on human vitamin D status and health. Therefore, long-term randomised controlled trials to evaluate the effect of 25(OH) D_3 fortified foods on vitamin D status are needed for both the general population and patients with certain conditions.

Key words

Vitamin D, 25(OH) D, fortification, biofortification, randomised controlled trial, dairy

Introduction

Vitamin D is a lipid soluble vitamin that acts as a hormone (Nair & Maseeh 2012), which generally refers to ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3) (Tripkovic *et al.* 2012). Vitamin D_2 and vitamin D_3 are produced by fungi and the skin of vertebrates, respectively (Wacker & Holick 2013). The role of vitamin D in musculoskeletal health is well established (Wolff *et al.* 2008). Recently, vitamin D deficiency has been suggested to be associated with several non-musculoskeletal health outcomes, such as cardiovascular disease, certain cancers and type 2 diabetes, although mechanisms are not clear (Wang *et al.* 2017). Vitamin D status is assessed by measuring the blood concentration of circulating 25-hydroxyvitamin D (25(OH) D) (Holick 2009). Widespread hypovitaminosis D is now acknowledged (Hilger *et al.* 2014), although there is some dispute about the thresholds for vitamin D deficiency and insufficiency (Spiro & Buttriss 2014). In the UK, vitamin D deficiency is defined as 25(OH) D <25 nmol/L (SACN 2016). The UK *National Diet and Nutritional Survey* (*NDNS*) reported that in 2008-2012 24% men and 21.7% of women (aged 19-64 years) had vitamin D deficiency (Bates *et al.* 2014). With seasonal variation, the prevalence of hypovitaminosis D in the UK was alarmingly high during winter and spring. A cross-sectional study conducted in the UK by Hypponen and Power (2007) reported that during the winter and spring months 25(OH) D concentrations were <25 nmol/L, <40 nmol/L and <75 nmol/L in 15.5%, 46.6% and 87.1% of participants, respectively. There are several additional contributors to hypovitaminosis D, such as skin pigmentation, sunscreen usage, and an increasingly indoor lifestyle, all of which reduce the cutaneous production of vitamin D (Holick 2004). Furthermore, vitamin D supplement can also contributes to vitamin D intake, however, uptake of supplements tends to be low (Hennessy *et al.* 2017; Datta *et al*. 2016). As a result, dietary intake of vitamin D has become more important than before (O'Mahony *et al.* 2011) and in recognition of this, in 2016, the UK Scientific Advisory Committee on Nutrition

(SACN) recommended the national population dietary of 10 μg vitamin D daily for everyone aged 4 years and older (SACN 2016). As there are a limited number of foods naturally enriched with vitamin D (such as egg yolk, oily fish and wild mushroom) (Schmid & Walther 2013), other strategies to improve vitamin D dietary intake are essential.

Vitamin D forms, metabolites and absorption

The two forms of vitamin D, D_2 and D_3, have similar chemical structures apart from vitamin D_2 having an additional methyl group and double bond (Hollis 1984). Humans and animals usually synthesise vitamin D_3 in the skin by converting 7-dehydrocholesterol in the epidermis to pre-vitamin D_3 in response to exposure to ultraviolet B radiation (UVB). Pre-vitamin D_3 then undergoes a temperature-dependent isomerisation to produce vitamin D_3 over approximately 3 days (Holick & Chen 2008). Vitamin D_2 and D_3, obtained from the diet, are absorbed with long-chain triglycerides in the small intestine and then incorporated into chylomicrons and transported via lymph to the circulation (Guo *et al.* 2018b).

After entering the blood circulation, vitamin D_2 and D_3 follow the same pathways to synthesise the biologically active form of 1, 25$(OH)_2$ D. There are two hydroxylation reactions: the first reaction occurs in the liver where vitamin D_2 and vitamin D_3 are hydroxylated to 25(OH) D_2 and 25(OH) D_3 by the vitamin D-25-hydroxylase; the second occurs in the kidney where 25(OH) D_2 and 25(OH) D_3 are converted to 1α,25$(OH)_2$ D_2 and 1α,25$(OH)_2$ D_3, respectively, by the 25-hydroxyvitamin D-1α-hydroxylase (DeLuca 1974).

Food sources and content of vitamin D

Vitamin D_2 and D_3 can be found in fungi (*e.g.* mushrooms) and animal-derived foods (*e.g.* eggs, oily fish), respectively (McCance & Widdowson 2015). In addition, there are significant quantities of the 25(OH) D metabolite in animal-derived foods (Ovesen *et al.* 2003). Previous

studies (Guo *et al.* 2017b; Lu *et al.* 2007; Phillips *et al.* 2011) have showed that the vitamin D concentrations of these foods can vary significantly between and within species (O'Mahony *et al.* 2011). For example, Phillips *et al*. (2011) collected and analysed the vitamin D_2 concentrations in 10 types of mushrooms from retail suppliers in the US, and reported that they were low (0.1-0.3 µg/100 g) in *Agaricus bisporus* (White Button, Crimini, Portabella) and Enoki, moderate in Shiitake and Oyster (0.4-0.7 µg/100 g), and high in Morel, Chanterelle, Maitake (5.2-28.1 µg/100 g). Furthermore, the vitamin D content of foods may relate to different production systems and the time of the year. For example, our study (Guo *et al.* 2017b) investigated eggs from three different production systems (organic, free range and indoor) over 5 months and showed a higher vitamin D_3 content in free range eggs (57.2 ± 3.1 µg/ kg) and organic eggs (57.2 ± 3.2 µg/ kg) compared with indoor eggs (40.2 ± 3.1 µg/ kg) (P <0.001). A seasonal effect on the vitamin D content of eggs has also been reported by others (Mattila *et al.* 2011a). The study of Lu *et al*. (2007) evaluated the vitamin D content of salmon, and found that farmed salmon had only ~ 25% of the vitamin D content of wild salmon and cooking may also cause detrimental loss of vitamin D. The study of Jakobsen & Knuthsen *et al*. (2014) investigated the loss/ retention of vitamin D during different cooking methods (frying, baking and boiling) in eggs and margarine. The results showed there was 39-45% retention of vitamin D content in eggs and margarine during baking in an oven for 40 minutes, while frying resulted in vitamin D retention of 82-84%. The author concluded that the loss/ retention of vitamin D during typical household cooking should be taken into account when calculating the dietary intake of vitamin D.

In general, there are two approaches to fortify foods with vitamin D: 1) 'direct fortification' by adding vitamin D into foods and 2) 'biofortification' of food by fortifying animal's diet with vitamin D (Cashman & Kiely 2016). For countries such as the UK where vitamin D fortification of foods is not mandatory (Kiely & Black 2012), populations have to rely on

dietary sources (including supplements) to maintain an adequate vitamin D status when there are limited sunlight. In the UK, the mean daily vitamin D dietary intake (excluding supplements) was 2.9 and 2.5 µg/day for men and women, respectively (*NDNS* 2008/2009-2011/2012; Bates *et al.* 2014)), which is far less than the current UK dietary reference nutrient intake (RNI) for vitamin D of 10 µg/day (SACN 2016). Therefore, approaches to increase vitamin D dietary intake have become necessary and urgent.

Comparative effectiveness of different forms of vitamin D at raising blood 25(OH) D concentrations

Vitamin D_2 and vitamin D_3

Blood 25(OH) D [the summation of 25(OH) D_2 and 25(OH) D_3] concentration is widely used as a biomarker of vitamin D status (SACN 2016). Early studies reported conflicting results on the relative effectiveness of dietary vitamin D_3 compared with vitamin D_2 for increasing serum/plasma 25(OH) D concentrations (Tripkovic *et al.* 2017). Tripkovic *et al*. (2012) conducted a systematic review and meta-analysis comparing the effects of dietary vitamin D_2 and vitamin D_3 on serum 25(OH) D concentrations in humans. Data were included from seven randomised controlled trials (RCTs) and the results showed that vitamin D_3 intake led to a greater absolute change in serum/plasma 25(OH) D levels from baseline than vitamin D_2, with a weighted mean difference of 15.23 (95% CI: 6.12, 24.34; Z=3.28; I^2=81%; P=0.001). Recently, a review by Wilson *et al*. (2017) summarised the evidence to date on the relative effectiveness of vitamin D_3 and vitamin D_2 at raising 25(OH) D concentrations and concluded that most RCTs showed that vitamin D_3 is more effective.

Vitamin D_3 and 25(OH) D_3

Of the few studies performed, most have found that the vitamin D metabolite 25(OH) D_3 given orally increases vitamin D status more efficiently than oral vitamin D_3, although no consensus has been established for the relative potency of 25(OH) D_3 and vitamin D_3 (Jakobsen 2007). Our recent review (Guo *et al.* 2018b) summarised the available evidence (Cashman *et al.* 2012; Catalano *et al.* 2015; Jetter *et al.* 2014; Navarro-Valverde *et al.* 2016) comparing 25(OH) D_3 with vitamin D_3 on serum or plasma 25(OH) D_3 concentrations, and concluded that the relative effectiveness of 25(OH) D_3 to vitamin D_3 ranged from 3.13 to 7.14. These variable results probably reflect differences in study designs and/or characteristics of the investigated subjects. In addition, evidence from available RCTs (Guo *et al.* 2018b) indicates that 25(OH) D_3 fortified dairy drink resulted in plasma 25(OH) D reach its peak significantly earlier than with vitamin D_3 fortified dairy drink. Thus, supplementation with 25(OH) D_3 might increase vitamin D status more efficiently and effectively than vitamin D_2 and vitamin D_3. Moreover, since the use of 25(OH) D_3 avoids the need for the liver to convert vitamin D_3 to 25(OH) D_3 it may be of particular value to patients with impaired liver function.

Food fortification with vitamin D

Direct fortification

In the US and Canada, several common foods, such as milk, orange juices, breakfast cereals, yogurts and cheeses are fortified with vitamin D (Holick *et al.* 2011). In Europe, vitamin D mandatory and voluntary fortification policies and practice vary from country to country (Spiro & Buttriss 2014). A meta-analysis was performed by Black *et al.* (2012), which included sixteen RCTs to evaluate the efficacy of vitamin D food fortification for improving vitamin D status. The results showed a mean intake of vitamin D of 11 µg/day from fortified foods (range 3-25 µg/day) increased serum/plasma 25(OH) D by 19.4 nmol/L (95% CI: 13.9-24.9), which corresponded to a 1.2 nmol/L (95% CI: 0.72, 1.68) increase in serum/plasma 25(OH) D for

each 1 µg ingested. Thus, vitamin D direct fortification could be an effective strategy to increase vitamin D status in the general UK population.

In the US and Canada, much of the vitamin D intake is from fortified foods (Fulgoni *et al.* 2011; Langlois *et al.* 2010). The major fortified foods contributing to vitamin D intake in these countries are fluid milk, ready-to-eat cereals and margarine (Calvo *et al.* 2004; Feldman *et al.* 2011). The study by Langlois *et al.* (2010) estimated vitamin D status among 5306 individuals aged 6- 79 years in the 2007- 2009 *Canadian Health Measures Survey* and showed that the mean 25(OH) D concentration was 67.7 nmol/L, and that 4% and 10% of the population had vitamin D deficiency (<27.5 nmol/L) and inadequacy (<37.5 nmol/L), respectively. In addition, subjects who consumed vitamin D fortified milk had higher 25(OH) D concentrations than non-consumers. In addition, voluntary fortification of foods with vitamin D has occurred in Finland since 2003 (Pilz *et al.* 2018), and the data from the *Finnish Health 2011 Survey* showed that mean serum 25(OH) D increased from 47.6 nmol/L in year 2000 to 65.4 nmol/L in 2011 (Jaaskelainen *et al.* 2017). However, a recent review (Calvo & Whiting 2013) questioned the adequacy of vitamin D fortified foods in the US and Canada to meet the needs of all race, gender and age groups. Furthermore, a review by Kiely *et al.* (2012) pointed out well-designed sustainable fortification strategies are needed to take account for diversity in food consumption patterns. In the UK, the food fortification policy was effective in preventing rickets in the 1950s; however, the mandatory vitamin D fortification policy was banned when over-fortification of some milk products led to cases of hypercalcaemia in young children (British Pediatric Association 1956). More research is needed to explore the safety of vitamin D fortification, including the range of products and doses of vitamin D added in each.

Biofortification

Biofortification of vitamin D is an alternative strategy to increase vitamin D intakes in countries and regions where policies and practices limit use of 'direct fortification'.

Our previous review provides an overview of recent vitamin D biofortification studies (Guo *et al.* 2018b), and found that the amount of vitamin D_3 and 25(OH) D_3 in eggs, fish and milk increases in response to vitamin D_3 supplementation of the diets of hens, fish and cows. However, evidence relating to 25(OH) D_3 supplementation of animals' diets is very limited, with the only available data for hens (Guo *et al.* 2018b). Interestingly, egg enrichment studies (Duffy *et al.* 2017; Mattila *et al.* 2011b) showed that supplementing hens' diets with 25(OH) D_3 results in an increase in the 25(OH) D_3 concentration, but not vitamin D_3, of the egg yolk. Thus, foods biofortified or fortified with either vitamin D_3 or 25(OH) D_3 are likely to have a variable effect on human vitamin D status (Mattila *et al.* 2011b).

Our recent milk biofortication study (Guo *et al.* 2018a) used a total of 60 dairy cows randomised to vitamin D_3 or 25(OH) D_3 dietary supplementing treatments, within the maximum permitted European Union (EU) vitamin D_3 concentration (2 mg/day vitamin D_3) for feed. The results showed that supplementing dairy cows' feed with 25(OH) D_3 significantly increased circulating plasma concentrations of 25(OH) D_3 in the cows. However, there was also no significant effect of the treatment on milk 25(OH) D_3 concentrations (P=0.193), the mean 25(OH) D_3 concentrations for non-fortified and 25(OH) D_3 dietary treatments were 869 and 1001 ng/kg, respectively. In addition, the vitamin D concentration (100-3,300 ng/kg) of the biofortified milk was negligible and far less than the current UK vitamin D recommended intake of 10 µg/day (SACN 2016). In the future, more studies are needed to explore which forms and doses of vitamin D added to animal diets, within the bounds of EU regulation (EC 2017; EFSA 2012), including those of fish, may have the greatest impact on human dietary quality.

Evidence from human intervention studies with 25(OH) D_3 fortified foods

Evidence of the effect of 25(OH) D_3 fortified food on increasing vitamin D status is limited. We were the first to compare the effects of dairy drinks fortified with either 20 µg 25(OH) D_3 or 20 µg vitamin D_3 on changes in human 24-hour vitamin D status (Guo *et al.* 2017a). The results showed plasma 25(OH) D_3 was significantly higher after the 25(OH) D_3 fortified dairy drink compared with the vitamin D_3 fortified dairy drink and control (non-fortified dairy drink), which was reflected in the 1.5-fold and 1.8-fold greater incremental area under the curve of plasma 25(OH) D_3 for the 0-8 hour response, respectively. However, we did not investigate the long-term effects of consuming the 25(OH) D_3 and vitamin D_3 fortified dairy drinks.

Hayes *et al*. (2016) conducted an 8-week RCT to compare the effects of consuming vitamin D_3 or 25(OH) D_3 biofortified eggs (7 per week for 8 weeks), obtained from feeding hens with the maximum concentration of vitamin D_3 or 25(OH) D_3 lawfully allowed in their diets, with a control treatment (≤ 2 commercial eggs/week), on winter serum 25(OH) D concentrations in healthy adults. At the 8 week follow-up in winter the vitamin D status of the subjects who consumed the vitamin D_3 or 25(OH) D_3 biofortified eggs was maintained [50.4 nmol/L (SD=21.4) and 49.2 nmol/L (SD=16.5) for vitamin D_3 and 25(OH) D_3 group, respectively], while the control group's vitamin D status significantly decreased over winter (-6.4 ± 6.7 nmol/L). In contrast with our study (Guo *et al.* 2017a), there was no significant difference between vitamin D_3 and 25(OH) D_3 biofortified egg consumption on the participants' serum 25(OH) D concentrations. The reason is unknown, but maybe because baseline vitamin D status (mean 46.2 nmol/L) was much higher than our study (mean 31.7 nmol/L), and vitamin D dose (3.5-4.5 µg/egg) for fortified eggs (Hayes *et al.* 2016) was only 20% of ours (20 µg/day) (Guo *et al.* 2017a)..

25(OH) D_3 supplementation and human health

As an alternative strategy to increase vitamin D status, it is possible that supplementation with 25(OH) D_3 may benefit human health more than with vitamin D_3, although the evidence is limited. A study of Bischoff-Ferrari *et al.* (2012) provided 20 µg/day of 25(OH) D_3 or vitamin D_3 to 20 healthy postmenopausal women over 4 months [mean baseline serum 25(OH) D concentration was 42 nmol/L]. The results showed 25(OH) D_3 supplementation resulted in a more immediate and sustained increase of serum 25(OH) D concentrations than vitamin D_3 supplementation. The mean 25(OH) D concentration increased to 221 nmol/L and 99 nmol/L for 25(OH) D_3 and vitamin D_3 supplementation, respectively. In addition, 25(OH) D_3 supplementation was found, on average, to result in a 2.8-fold increased odds of maintained or improved lower extremity function (OR=2.79, 95% CI: 1.18-6.58), and a 5.7 mmHg decrease in systolic blood pressure compared with vitamin D_3 (P=0.0002). In another study, Jean *et al.* (2008) provided 10-30 µg/day 25(OH) D_3 to haemodialysis patients for 6 months, and the results showed vitamin D status increased from 30 nmol/L to 126 nmol/L, and 25(OH) D_3 supplementation corrected their excess bone turnover.

A review by Brandi & Minisola (2013) summarised the available evidence in this area and concluded that for populations that have specific conditions (such as long-lasting vitamin D osteomalacia, liver failure, latrogenic inhibition of liver 25-hydroxylases, inactivating mutations of genes encoding liver 25-hydroxylasese, kidney failure with elevated PTH, nephrosis, transplanted patients, male hypogonadism), supplementation with 25(OH) D_3 may prove to be preferable to vitamin D_3. The reasons might be because 25(OH) D_3 avoids the need for hepatic metabolism of vitamin D_3 to 25(OH) D_3, which results in 25(OH) D_3 more quickly entering the blood circulation (Holick 1995; Ross *et al.* 2011).

Currently, vitamin D_2 and vitamin D_3 are legally permitted to be added to foods, but addition of 25(OH) D_3 is not (EC No 1925/2006). Future studies should focus on better defining the

long-term effects of 25(OH) D_3 fortified foods on vitamin D status and human health, compared to vitamin D_3 and vitamin D_2.

Conclusions and future directions

Vitamin D deficiency and insufficiency have become global problems, especially where sunlight is limited by latitude, cultural reasons or lifestyle (Hilger *et al.* 2014). The UK government advisory committee, SACN, recommends an intake of 10 µg/day of vitamin D for the UK general population (SACN 2016). However, it is a great challenge to meet this recommendation from solely natural dietary sources and uptake of supplements tends to be low. Two potential strategies to increase vitamin D content of food are direct fortification and biofortification via animal diet supplementation. However, evidence from RCTs is limited on the effect of vitamin D fortified foods on human vitamin D status and human health. The available evidence suggests that the vitamin D metabolite, 25(OH) D_3, might be more efficient than vitamin D_2 and D_3 at raising serum or plasma 25(OH) D_3 concentrations in both general healthy subjects and clinical patients. In addition, 25(OH) D_3 may have an advantage of improving the health of certain clinical patients, although the evidence for this is limited. Therefore, 25(OH) D_3 fortified foods (including direct fortification and biofortication) should be further explored in the future, and additional RCTs should be conducted to investigate the effect of 25(OH) D_3 fortified foods on vitamin D status and human health in both healthy subjects and clinical patients.

Conflict of interest

The authors have no conflict of interest to disclose.

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