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Impact of whole egg consumption and maintenance of vitamin D homeostasis in disease

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Impact of whole egg consumption and maintenance of vitamin D homeostasis in disease

by

Samantha Pritchard

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Nutritional Sciences

Program of Study Committee:
Kevin Schalinske, Co-major Professor
Matthew Rowling, Co-major Professor
Donald Beitz
Peter Clark
Lorraine Lanningham-Foster
Manju Reddy

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2018

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ABSTRACT

Vitamin D deficiency has been reported to affect 30% of the American population, owing to poor dietary intake and insufficient sun exposure. Pregnant and lactating women, the elderly, and individuals with increased skin melanin pigmentation are at especially high risk for developing poor vitamin D status. The classical, hormonal actions of vitamin D related to mineral metabolism and skeletal health are well-established. More recently, however, evidence suggests that vitamin D deficiency increases the risk for chronic diseases such as obesity, diabetes, cancer and autoimmune disorders. Dietary eggs represent a natural whole food source of vitamin D₃ as well as 25-hydroxycholecalciferol (25D), the vitamin D metabolite that represents an individual's status. The objective of the studies described in this dissertation were to determine if a whole egg-based diet could maintain vitamin D status in 1) Zucker Diabetic Fatty (ZDF) rats, a well-characterized animal model of type 2 diabetes (T2D), 2) Streptozotocin-induced type 1 diabetic (T1D) rats, and 3) in Sprague Dawley rats with dextran sulfate sodium-induced colitis.

In the first study described in this dissertation, male ZDF rats (n = 12) and their lean counterparts (n = 12) were randomized to one of two dietary treatment groups, a casein- or whole egg-based diet, for 8 weeks. Both diets contained 25 µg cholecalciferol/kg diet, provided by the vitamin mix. The whole egg-based diet contained an additional 12.6 µg cholecalciferol/kg diet; thus, the whole egg-based diet provided a total of 37.6 µg cholecalciferol/kg diet. Both diets provided protein at 20% (w/w) and contained the same lipid content by the addition of corn oil to the casein-based diet to match the lipid contribution by the addition of whole egg. Whole egg consumption

attenuated both hyperglycemia and hypertriglyceridemia, as well as reduced weight gain in ZDF rats compared to casein-fed diabetic rats. Circulating 25D was lower in casein-fed ZDF rats compared to lean controls; however, ZDF rats fed whole egg exhibited the same circulating 25D concentrations as casein-fed lean rats. These data suggest that dietary whole egg can attenuate metabolic anomalies, as well as maintain normal circulating 25D concentrations in T2D rats.

This second study described in this dissertation compared whole egg consumption to supplemental cholecalciferol with respect to vitamin D balance, body weight gain, and body composition in T2D rats. Male ZDF rats (n = 24) and their lean controls (n = 24) were randomly assigned to one of 3 dietary treatment groups: a casein-based diet (CAS), a dried whole egg-based diet (WE), or a casein-based diet containing supplemental cholecalciferol (CAS+D) at the same level of cholecalciferol provided by the dried whole egg-based diet (37.6 µg/kg diet). All diets provided protein at 20% (w/w) and contained the same lipid contribution by the addition of whole egg. Rats were fed their respective diets for 8 weeks. Weight gain and percent body fat were reduced by approximately 20% and 11%, respectively, in ZDF rats fed WE compared to ZDF rats fed CAS or CAS+D. ZDF rats fed CAS had 21% lower serum 25D concentrations than lean rats fed CAS. In ZDF rats, WE consumption increased serum 25D concentrations 130% compared to CAS, whereas consumption of CAS+D increased serum 25(OH)D concentrations 35% compared to CAS. Our data suggest that dietary consumption of whole egg is more effective than supplemental cholecalciferol in maintaining circulating 25D concentrations in T2D rats. Furthermore, whole egg consumption reduced weight gain in obese T2D rats, without effect on body weight in a lean phenotype. These data may support new

dietary recommendations targeting obesity and prevention of vitamin D insufficiency in T2D.

The objective of the third study was to investigate the impact of whole egg consumption in T1D rats. Male Sprague Dawley rats were randomly assigned to either a casein-based (n = 12) or a whole egg-based diet (n = 6) for 32 days. Both diets provided protein at 20% (w/w) and contained the same total lipid content by the addition of corn oil to the casein-based diet to match the lipid contribution by the addition of whole egg. The vitamin mix in both diets provided 25 µg cholecalciferol/kg diet. The whole egg-based diet contained an additional 12.6 µg cholecalciferol/kg diet; thus, the whole egg-based diet provided a total of 37.6 µg cholecalciferol/kg diet. On day 26, all rats in the whole egg-based diet group and half of the rats on the casein-based diet received a streptozotocin injection to induce T1D for the final week of the experimental period. Whole egg consumption attenuated polyuria, proteinuria and renal hypertrophy in T1D rats. These data suggest that dietary intervention with whole egg may offer renal protection in T1D. Understanding the mechanism underlying the nephroprotective effect of dietary whole egg will be a focus of future work.

The goal of the fourth and final study presented in this dissertation was to investigate the impact of whole egg consumption in dextran sulfate sodium (DSS)-induced colitis. In an initial dose response study, male Sprague Dawley rats (N= 24) were maintained on a casein-based diet for 5 weeks and randomly assigned to 0, 3, 4 or 5% DSS-treated drinking water for the final 7 days of the study. Serum 25D concentrations exhibited a dose-response decrease with respect to increasing DSS concentrations. In a follow-up study, Sprague Dawley rats (N=36) were randomly assigned to a casein-

whole egg- or a casein-based diet containing supplemental cholecalciferol at the same level of cholecalciferol provided by the dried whole egg-based diet (37.6 µg/kg diet). All diets provided protein at 20% (w/w) and contained the same lipid contribution by the addition of whole egg. Rats were fed their respective diets for 8 weeks. For the final 7 days of the study, half of the rats in each group were given 3.5% DSS-treated drinking water, based on the results of initial dose response study. Serum 25D concentrations were the same between rats fed the casein-based diet and casein-based diet containing supplemental cholecalciferol. Rats fed a whole egg-based diet, however, exhibited increased serum 25D concentrations that were significantly higher than rats in either the other dietary intervention groups, regardless of colitis status. These data suggest that whole egg consumption may be more effective than supplemental cholecalciferol at increasing circulating 25D concentrations in experimental colitis.

The studies described in this dissertation indicate that whole egg consumption is an effective means of increasing serum 25D concentrations in animal models of diabetes and inflammatory bowel disease. Future dose response studies are needed to identify the specific quantity of egg consumption that is efficacious with respect to influencing vitamin D status and disease complications.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Vitamin D is a fat-soluble vitamin that is acquired by endogenous synthesis via the action of sunlight exposure on the skin. It is also obtained in the diet as vitamin D₂, commonly found in plants, or as vitamin D₃, provided by foods of animal origin and supplementation (1, 2). Vitamin D acquired in either manner is physiologically inert and once absorbed must undergo two hydroxylations in the body for activation. The first occurs in the liver and converts vitamin D to 25-hydroxyvitamin D (25D), the gold standard measurement of vitamin D status for an individual. The second occurs primarily in the kidney and forms the biologically active 1,25-dihydroxyvitamin D (1,25D) (3, 4). Population trends in the U.S. indicate that circulating 25D concentrations are declining and may be the result of a combination of decreased dietary intake, limited sunlight exposure, compromised vitamin D absorption, or renal insufficiency resulting in decreased conversion of 25D to 1,25D (5). Additionally, pregnant and lactating women, the elderly and individuals with increased skin melanin pigmentation are at especially high risk for developing poor vitamin D status (6). In infants and children, vitamin D deficiency causes rickets, a disease characterized by the failure of cartilage to mature and mineralize into bone, leading to the development of soft bones and skeletal deformities. In adults, deprivation of vitamin D manifests into osteomalacia that is characterized by defects in bone mineralization caused by alterations in calcium and phosphorous absorption and excretion, resulting in weak bones (6). The classical, hormonal actions of vitamin D related to mineral metabolism and skeletal health are well-established. More recently, however, evidence has demonstrated an

association between vitamin D deficiency and a variety of chronic diseases such as obesity, diabetes, cancer, and autoimmune disorders (7).

It is well established that dietary whole eggs contain a variety of nutrients, particularly compared to other animal products (8). They have long been promoted for their high-quality protein and high nutrient density-to-energy ratio, providing several nutrients in excess of its caloric content, particularly vitamin D₃ and 25D (8-10). There are no specific guidelines for routine egg consumption. As part of a balanced diet, it is suggested to incorporate whole egg intake together with a variety of fresh produce, whole grains and lean meats (8). Egg consumption has faced a controversy for several decades as a result of the initial observational studies that established a link between dietary cholesterol and cardiovascular disease risk (11, 12). A growing body of literature, however, demonstrates the contrary. Studies show that as much as one egg per day is not detrimental to health (13, 14). Apart from the nutritional benefits, fundamentally, eggs are inexpensive and easy to prepare (9).

The objectives of the present research were to determine if a whole egg-based diet could maintain vitamin D status in 1) Zucker Diabetic Fatty (ZDF) rats, a well-characterized animal model of type 2 diabetes (T2D); 2) Streptozotocin-induced type 1 diabetic (T1D) rats; and 3) in Sprague Dawley rats with dextran sulfate sodium-induced colitis.

Dissertation Organization

This dissertation consists of seven chapters with a general introduction, literature review, four manuscripts, and an overall conclusion. The first manuscript titled, “Whole egg consumption prevents diminished serum 25-hydroxycholecalciferol concentrations in type 2 diabetic rats” has been published in the *Journal of Agriculture and Food Chemistry*. This

manuscript reported the maintenance of vitamin D homeostasis and reduced weight gain in a T2D animal model. The work presented in the second manuscript compared whole egg consumption to supplemental cholecalciferol with respect to vitamin D balance, weight gain, and body composition in T2D rats and was published in the *Journal of Nutrition*. The work presented in the third manuscript investigated the impact of whole egg consumption in a T1D animal model and was published in the *Journal of Agriculture and Food Chemistry*. The final manuscript evaluated the impact of whole egg consumption in dextran sulfate sodium-induced colitis and has been prepared for submission to the *Journal of Agriculture and Food Chemistry*. All literature cited is based on the format of the *Journal of Nutrition* and is listed at the end of each chapter. The final chapter describes the overall results and future directions of the research presented in this dissertation.

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CHAPTER 2. LITERATURE REVIEW

Diabetes Mellitus

The number of individuals around the world with diabetes has nearly quadrupled since 1980, affecting approximately 422 million people today (1). In the United States, it is estimated that 30 million children and adults are living with diabetes (2). The disease is characterized by hyperglycemia caused by abnormalities in the secretion of insulin, action of insulin, or both. The criteria for diagnosis is as follows: fasting blood glucose concentration ≥ 126 mg/dL, hemoglobin A1c (HbA1c) $\geq 6.5\%$, 2-hour plasma glucose ≥ 200 mg/dL following an oral glucose tolerance test, as well as presentation of classic hyperglycemia symptoms. An individual is said to be diabetic if they satisfy any of the above criteria (3). Chronic, uncontrolled hyperglycemia is associated with macrovascular and microvascular complications including atherosclerosis, nephropathy, retinopathy and neuropathy. Studies have demonstrated that increased oxidative stress, secondary to prolonged elevated blood glucose, can contribute to the pathogenesis of these severe vascular complications, and includes the formation of free radicals, production of advanced glycation end products, as well as activation of the polyol pathway (4-7). The primary treatment goal for diabetes is to maintain glucose homeostasis with the intention of minimizing onset of macro- and microvascular complications (2).

Type 1 Diabetes (T1D)

T1D is the result of an immune-mediated destruction of insulin-producing pancreatic β -cells. Historically, T1D was considered a disorder in children and adolescents. Though onset typically occurs in preadolescence, it is now known that T1D can be diagnosed well

into adulthood (8). Upon diagnosis, the hallmark symptoms include polyphagia, polydipsia, and polyuria, and may also include hyperglycemia, weight loss or ketonemia (3). The etiology of T1D remains poorly understood, owing to the complex interaction between the environment, genetics, and the immune system. Generally, it is agreed upon that genetically susceptible individuals with a fixed number of β -cells are exposed to an environmental trigger, which induces β -cell autoimmunity (9, 10). The immune response is characterized by the development of islet reactive autoantibodies, up-regulation of interferons, as well as the recruitment of other pro-inflammatory cytokines. This process leads to the activation of autoreactive CD8 T-cells capable of destroying β -cells (10). The proliferation and migration of CD8 T-cells into the pancreas, with CD4 T-cells and B-cells, results in a progressive loss in insulin secretory function (9, 11). CD8+ T-cells are the most predominant population identified in insulinitis, suggesting a potential pathogenic mechanism. Furthermore, insulinitis is only present in β -cells, implying that the islet infiltration is a β -cell-driven process (12). One of the candidates reported to be associated with impaired T-cell tolerance induction during islet autoimmunity onset is the Human Leukocyte Antigen (HLA) complex. HLA is a cell surface protein responsible for immune system regulation. It is estimated that HLA provides 60% of the overall genetic susceptibility in T1D. There are three classes of HLA genes. Class II genes are said to have the strongest association with T1D because they encode for molecules that contribute to antigen presentation (13). With that said, more remains to be determined about the risk factors that contribute to HLA dysregulation and T1D pathogenesis.

MicroRNAs (miRNAs) are a class of small noncoding RNAs that regulate gene expression by partially pairing to the 3', 5' untranslated regions of their target mRNAs,

resulting in translation repression and/or transcript degradation. Growing evidence suggests that miRNAs play a crucial role in T1D pathogenesis, including immune system activation and β -cell function (14). In a recent study by Serr et. al (15), miRNA181a was linked to impaired tolerance induction and autoimmune activation, via nuclear factor of activated T-cells 5 (NFAT5). In an in vitro experiment using isolated T-cells from children with early stage islet autoimmunity, the investigators determined that enhancing miRNA181a activity increases NFAT5 expression and inhibits Forkhead box protein P3 positive (FoxP3+), preventing the proper development of T-regulatory cells. In a follow-up study using a humanized mouse model, the investigators found that blocking miRNA181a or NFAT5 reduced murine islet autoimmunity (15).

In recent years, the influence of the gut microbiota on immune responses has been the subject of a number of studies (16, 17). With respect to the risk of developing T1D, evidence from in vivo studies using T1D animal models suggests that, even prior to T1D onset, the microbial population of the gut differs between rodents that develop diabetes and rodents that remain healthy (18, 19). Wen et. al (20) reported that specific pathogen-free non-obese diabetic mice lacking MyD88 protein, the innate immune signaling molecule that identifies microbial stimuli, are protected from the development of T1D (20). While additional research is needed to investigate the interaction of the intestinal microbes with the innate immune system as a factor modifying T1D risk, these studies highlight the prospect of a targeted intervention for individuals with T1D.

Because of its role in the prevention of acute and long-term T1D complications, glycemic control is the foundation for managing the disease. Treatment approaches are individualized and focus primarily on managing intake of carbohydrates and integrating an

insulin regimen to maintain blood glucose concentrations (21). New technologies can assist T1D individuals with optimizing glycemic control and decreasing the risk of severe hypoglycemia. These include insulin pumps, continuous glucose monitors (CGM), and sensor-augmented pumps, which is a “smart” device that combines the technologies of a traditional insulin pump with CGM and therapy management software that wirelessly transmits glucose readings to the person wearing the device (22). Additionally, pancreatic islet cell transplant is a current experimental procedure that involves transferring islets from the pancreas of an organ donor into a T1D individual whose blood glucose concentrations are difficult to manage (23). The transplant goals are to assist patients with normalizing blood glucose concentrations and to decrease the number of hypoglycemic events that patients are unaware of (23).

Type 2 Diabetes (T2D)

T2D accounts for 90-95% of all global diabetes cases (24). It is estimated that by the year 2040, 642 million people will be living with T2D secondary to overnutrition, a sedentary lifestyle and subsequent weight gain. The primary risk factors for T2D include obesity, physical inactivity, family history of T2D, history of gestational diabetes and non-white race and/or ethnicity (25). T2D is generally characterized by hyperglycemia and hyperinsulinemia. In contrast to T1D, the cause of hyperglycemia is more complex in T2D. Individuals with T2D continue to produce insulin, though the metabolism of glucose, in response to insulin as well as secretion of insulin, is abnormal (26).

In a healthy person, insulin secretion is triggered when glucose concentrations are increased in the postprandial state. The rise in extracellular glucose stimulates glucose uptake in the pancreatic β -cell, via glucose transporter-2 (GLUT2), where glucose is oxidized,

leading to a rise in ATP (27). The rise in ATP causes depolarization of the K^+ channel, which activates the voltage-gated Ca^{2+} in the plasma membrane, promoting an influx of Ca^{2+} into the β -cell (27). This rise in intracellular Ca^{2+} concentrations triggers exocytosis of insulin-containing secretory granules, from the endoplasmic reticulum (ER) to the plasma membrane, and subsequent release of insulin into the portal blood (28). The insulin signaling cascade begins when insulin binds to its receptor, activating the tyrosine kinase domain on the intracellular beta subunits. The activated receptor transduces its signals to downstream effectors, promoting tyrosine phosphorylation of several substrates, including the insulin receptor substrate (IRS) and phosphatidylinositol 3-kinase (29). In peripheral tissues, glucose transporter-4 (GLUT4) is recruited from the cytosol to the plasma membrane to facilitate glucose uptake. In T2D, insulin signaling is impaired. It has been suggested that serine, instead of tyrosine, phosphorylation of IRS-1 may be a potential cause of insulin resistance, due to the disruption in the insulin signaling cascade (27). As hyperglycemia persists, β -cells proliferate in order to compensate for the high demand of insulin. The ability of the ER to maintain insulin processing from pro-insulin results in activation of the unfolded protein response leading to β -cell stress and eventual apoptosis. Ultimately, there is a decrease in insulin release and exacerbation of the hyperglycemic state (29).

Overweight and obesity are also believed to be a key component in the onset of insulin resistance in T2D (30-33). While the exact mechanisms are not fully understood, perturbations in the immune-metabolic cross-talk have been implicated. Hotamisligil and colleagues (34) were among the first to establish a role for inflammation in obesity, demonstrating increased adipose tissue expression of tumor necrosis factor-alpha (TNF- α) in human adipocytes (34). Since then, other pro-inflammatory cytokines including interleukin-1

(IL-1) and interleukin-6 (IL-6), in addition to TNF- α , have been shown to disrupt insulin receptor signaling (31). Specifically, under positive energy balance, as body weight increases, excess energy is stored in the adipocyte as triglycerides, leading to adipocyte hypertrophy (32). As adipocyte storage reaches its threshold, there is an infiltration of macrophages. The adipocyte, along with the resident macrophages, will trigger the secretion of these pro-inflammatory cytokines as a mechanism to prevent lipotoxicity by decreasing cell mass. As a result, there is an increase in lipolysis and release of free fatty acids into the circulation, leading to the development of hypertriglyceridemia, a hallmark sign of T2D in newly diagnosed patients. Because the adipocyte fails to expand and adipogenesis is compromised, ongoing positive energy balance is coupled to ectopic lipid accumulation in the liver and muscle, further promoting local inflammation and insulin resistance (31, 32).

Through lifestyle and diet modifications, studies have demonstrated a significant reduction in the incidence of T2D with a combination of medical nutrition therapy, regular exercise, and psychosocial care. This suggests that, unlike T1D, T2D is largely a preventable disease (35). Evidence provided by the Diabetes Prevention Program (DPP) showed a 58% reduction in T2D, over a 3-year period, following an intensive lifestyle intervention (36). Follow-up studies focused on lifestyle interventions have demonstrated a 34-43% sustained reduction in the rate of conversion from prediabetes to fulminant T2D over 7-20 years (37-39). Treatment and management of T2D are not unlike prevention strategies. While glucose homeostasis remains a primary focus in the management of T2D, adoption of healthy lifestyle habits, blood pressure control and pharmacotherapy for dyslipidemia have collectively been shown to reduce hyperglycemia and decrease the onset and progression of vascular complications (40).

Inflammatory Bowel Disease (IBD)

According to the Centers for Disease Control and Prevention, in 2015, one-third of the adult population in the U.S. had been diagnosed with IBD (41), a chronic, relapsing inflammatory condition of the gastrointestinal (GI) tract that consists of two sub-types: Crohn's disease (CD) and ulcerative colitis (UC). Clinically, UC is characterized by superficial mucosal inflammation of the colon, rectal bleeding, diarrhea as well as abdominal pain (42). In contrast, CD may affect any portion of the GI tract, from the mouth to anus, and is characterized by a discontinuous and ulcerous transmural inflammation often involving the ileocecal region and may even lead to stricturing or fistulizing of the GI tract. Symptoms of CD include abdominal pain, fever, bloody or non-bloody diarrhea, and weight loss (43). The exact cause of IBD remains unclear; however, it is thought to be due to the interplay of a person's genetics, immune response, gut microbiome and the environment that result in an inappropriate and exacerbated immune response against commensal flora in those who are genetically susceptible (44).

The surface area of the intestinal epithelium is estimated to be 100 m² and is lined by a single layer of intestinal epithelial cells (IEC), serving as a physical barrier to luminal contents (45). IECs are made up of several specialized cell types, each with distinct functions that, together, support intestinal homeostasis by responding to signals provided by commensal microbiota and local leukocyte populations (46). Some of these distinct functions of IECs include production and secretion of compounds that influence microbial colonization, sampling of the intestinal microenvironment, sensing bacteria that may benefit or harm the host, and induction of immune responses. Additionally, the intracellular spaces between cells are linked together by junctional complexes. These complexes connect the

internal and external environment and are responsible for regulating the passage of ions and solutes (47). Apical tight junction (TJ) proteins are a critical component to the maintenance of intestinal epithelial barrier function, as they span the space between the apical and basolateral membranes and are a key factor in paracellular permeability. The transmembrane TJ proteins include occludins, tricellulin, claudins and junctional adhesion molecules. While peripheral membrane TJ proteins comprise of zona occludens and cingulin (47). Disruption of any one of these fundamental features of intestinal homeostasis, IEC or TJ proteins, may compromise the integrity of the intestinal epithelium and lead to a local immune response (46).

Several environmental factors have demonstrated a significant influence on the risk for IBD development and progression, including smoking, diet, antibiotic use, and social stress (48). Smoking continues to be the most widely studied environmental factor associated with IBD, though it increases the risk for CD while conferring a protective effect against UC (49). Studies demonstrate that patients with UC, who are also heavy smokers, exhibit an improvement in disease severity and a reduction in surgical intervention needs (48-50). By contrast, current smokers have a 2-fold increased risk of CD compared to non-smokers (49, 51).

The most consistently described dietary association with IBD has been the intake of soluble fiber. Generally, consumption of fruits, vegetables and whole grains has been shown to be inversely associated with CD and UC (52, 53). Consumption of a Westernized diet, one that is high in red meat intake, refined sugars and saturated fat and low intake of fiber, particularly fruits and vegetables, is associated with an increased IBD risk (53-57). There are also limited data on whether specific dietary factors impact disease flare. Data from patient

surveys exhibits a diverse response regarding specific trigger foods (58, 59). The heterogeneity suggests two postulations, either the non-existence of single food patterns that trigger disease relapse, or an underlying genetic predisposition, which may influence susceptibility to dietary constituents. Rigorous studies of dietary risk factors are needed to support dietary recommendations during periods of relapse (48).

Another environmental factor that has been implicated in IBD risk is antibiotic use and the consequent alteration of commensal flora. In a nested case-control study of 36 children diagnosed with IBD compared with databased-matched controls, 58% of the IBD group was reported to have used antibiotics in the first year of life compared to 39% in the control group (60). A follow-up study in adults reported a similar trend, where antibiotic use 2-5 years prior to diagnosis was more common in the IBD group compared to the adults in the control group. The same study also identified a dose-dependent effect with increasing use of antibiotic prescriptions in IBD cases (61). On the contrary, antibiotics are widely used to manage IBD activity and relapse (48, 62, 63).

Some studies have shown that stress is associated with higher incidences of relapse in IBD (64, 65). Because the hypothalamus-pituitary-adrenal (HPA) axis and the immune system work in tandem when the body encounters a stress-induced situation, it is thought that disruptions to the HPA axis-immune system crosstalk could lead to disorders characterized by inflammation due to abnormal responses to stressful stimuli (66). However, in an evidenced-based literature review by Abegunde and colleagues, little evidence was found to support an association between stress and increased IBD incidence (67).

The genetics of IBD are complicated and may be polygenic (44). Genome-wide association studies suggest that improper function of innate and adaptive immunity

contribute to the onset of IBD (68-71). To date, an estimated 215 genetic loci have been identified (72). The majority of susceptibility variants are associated with both UC and CD, while others are exclusive to each disorder. In general, the IBD-associated loci are enriched for genes involved in intestinal epithelial barrier function, T-cell function, cytokine production and autophagy, among others (73-75). A variant in the Muc2 gene confers IBD susceptibility in humans. Muc2 encodes for mucin, a primary component of the protective mucous layer that lines the intestine (44). In a study using Muc2 knockout mice, researchers determined that, by 5 weeks of age, Muc2-deficient mice demonstrated clinical colitis symptoms which were exacerbated as the mice aged. Analysis of colonic tissue exhibited mucosal thickening, increased proliferation and superficial erosions (76). A frameshift mutation to nucleotide-binding oligomerization domain containing 2 (NOD2) was the first susceptibility gene identified for CD (77). NOD2 is an intracellular pattern recognition receptor and activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), making it responsive to bacterial lipopolysaccharides. A genetic defect in NOD2 leads to increased inflammation due to impaired bacterial clearance. Moreover, a mutation in NOD2 has also been shown to result in suppression of the production of the anti-inflammatory cytokine interleukin 10 (IL-10) (78, 79).

Autophagy is a key process required for intracellular homeostasis following infection, mitochondrial damage or ER stress, and as such has a crucial role in cell-intrinsic defense against intracellular infections (46). The T300A disease-risk allele of the autophagy gene ATG16L1 is associated with an increased risk of CD. Patients with CD who are homozygous for the T300A mutation possess Paneth cell abnormalities, suggesting that the secretory granule pathway is impaired (80). Similarly, in a hypomorphic mouse model of the

ATG16L1 mutation, Paneth cells also exhibit cell granule abnormalities comparable to humans with the ATG16L1 gene variant. These hypomorphic ATG16L1 mice also demonstrated an increased susceptibility to dextran sulfate sodium-(DSS) induced colitis, though they did not develop colitis spontaneously (81).

The immune system plays a critical role in maintaining homeostasis with resident microbial communities. Likewise, resident bacteria profoundly influence mammalian immunity, ensuring that the reciprocal host-microbial relationship is maintained. (82). The gut microbiota is controlled by epithelial and immune cells via mucus, defensins and immunoglobulin-A (IgA), for example. Equally, intestinal immunity is regulated by the microbiota, as certain microbes, such as segmentous filamentous bacteria, Clostridia and Bacteroides fragilis, have been shown to favor growth of different lymphocyte subsets promoting the induction of regulatory T-cells and T-helper cells (82). Both UC and CD are characterized by dysbiosis. In an analysis comparing the fecal microbiota profiles between UC and CD, the fecal microbial communities of IBD patients were different from those of healthy individuals (83). Intestinal samples from individuals with IBD demonstrate an increase in abundance of Bacteroidetes and Proteobacteria and a decrease in abundance of Firmicutes, as well as a decrease in microbial diversity (84-86). An important question is whether IBD-associated disturbance in gut microbial communities is a primary or secondary phenomenon (87). Evidence supports the hypothesis that the microbiota is influenced by the host's genotype, which would be a primary factor in IBD pathogenesis (88). On the contrary, there is surmounting clinical and experimental data that suggests that infections, antibiotics, drugs and diet, among other environmental factors, can induce dysbiosis (89).

For individuals with acute IBD, the treatment goal is to improve quality of life and induce clinical remission of symptoms including colonic inflammation, rectal bleeding, diarrhea and abdominal pain (90). Once achieved, the goal is to maintain remission. Pharmacological intervention is the primary therapy for maintenance of IBD remission. The choice of pharmacological agents depends heavily on disease severity and location in the GI tract. Anti-inflammatory drugs are prescribed first and may include corticosteroids and aminosalicylates. The second line of treatment involves immune system suppressors (90). The most common class prescribed for UC and CD are TNF- α inhibitors, Infliximab and adalimumab. Both drugs are monoclonal anti-TNF- α antibodies that bind TNF- α and inhibit its pro-inflammatory effects in the intestine (90, 91). Evidence also supports the use of antibiotics as an effective adjuvant therapy in IBD. Prescriptions for antibiotics have been used to manage IBD-induced dysbiosis, as well as to reduce incidence of infections (92). Related, because of the role that intestinal microbiota is thought to play in IBD pathogenesis, fecal microbiota transplant (FMT) has been used for the management of IBD with some positive outcomes reported. In a systematic review summarizing 17 studies, 76% of IBD patients reported cessation of medications and reduction in symptom severity, while 63% reported disease remission (93). Larger randomized control studies are necessary, however, to evaluate the efficacy of FMT in the management and treatment of IBD (92). Dietary recommendations for individuals with IBD are personalized. Individuals are instructed to use a diary to monitor their food intake and symptoms. This approach allows the patient to identify specific triggers and empower them to make modifications as needed. Further controlled studies are necessary to create evidence-based dietary guidelines for patients with UC or CD (94).

Vitamin D

Biosynthesis and Metabolism

'Vitamin D' refers to the parental vitamin D acquired by either one of two sources, the first, endogenously by the action of sunlight exposure on the skin. Specifically, ultraviolet B (UVB) photons penetrate the epidermis and photolyze 7-dehydrocholesterol to previtamin D₃, which then isomerizes to vitamin D₃, is absorbed in the capillary bed and enters into the circulation (95, 96). Alternatively, vitamin D is also obtained in the diet as either vitamin D₃, provided by foods of animal origin, or vitamin D₂, commonly found in plants. Because vitamin D is fat soluble, it is incorporated into micelles along with other dietary fat, which then passively diffuses into the intestinal epithelium. In the enterocyte, vitamin D is incorporated into chylomicrons, which first enters the lymphatic system and finally the circulation (97).

Circulating vitamin D is transported to the liver via vitamin D binding protein (DBP). In the liver, 25-hydroxylase (CYP2R1) functions to hydroxylate vitamin D₃ to 25-hydroxyvitamin D₃ (25D). 25D is secreted into the circulation, binds to DBP and is then transported to the kidney, the primary tissue site for synthesis of the biologically active hormonal form of vitamin D, 1,25-dihydroxyvitamin D₃ (1,25D) (98, 99). DBP is a ligand of cubilin and megalin, two membrane-associated proteins, in the renal proximal tubule, that function together with the intracellular adaptor protein, disabled-2 (Dab2), to facilitate endocytic uptake of 25D. Thus, these receptors are the major means by which 25D is targeted to the kidney (99). Upon internalization into the proximal tubule, 25D can then either be converted to 1,25D by 25-hydroxyvitamin D α -hydroxylase (CYP27B1) or reabsorbed into circulation (Figure 1).

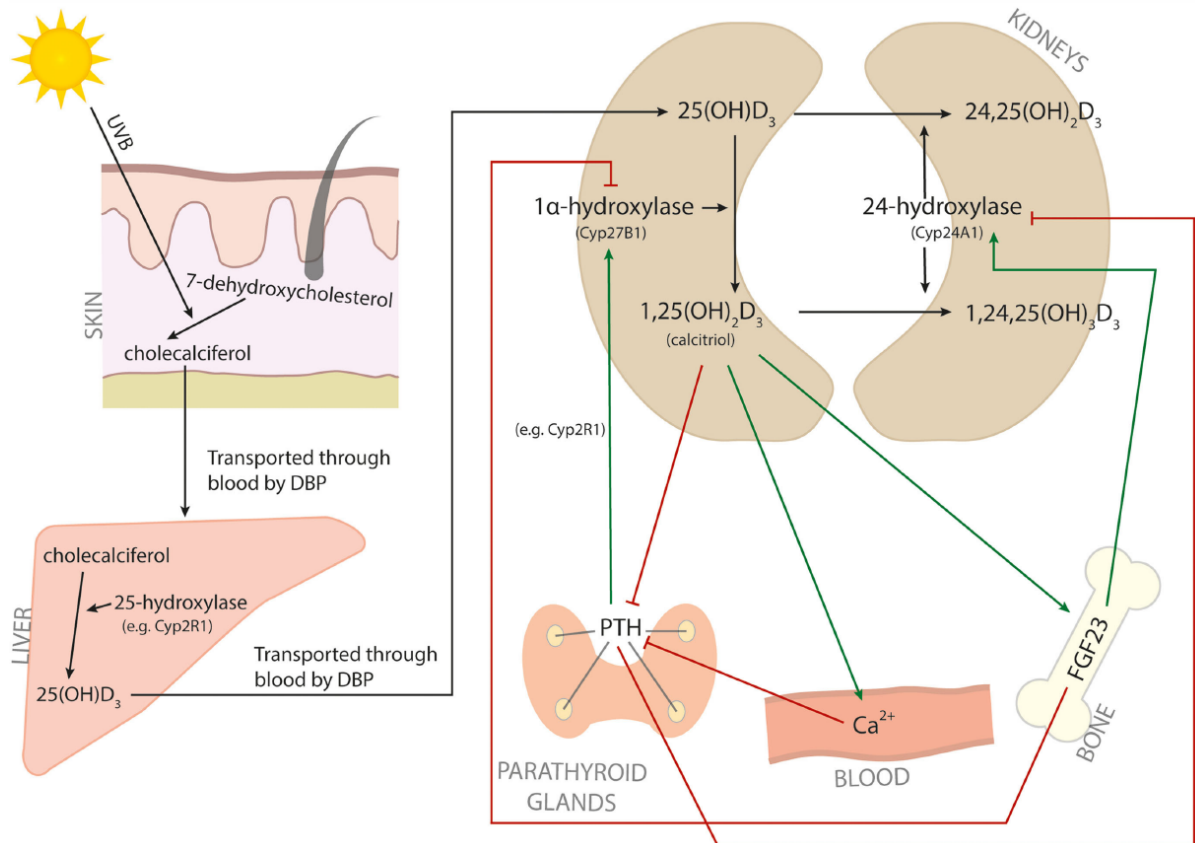


Figure 1. Vitamin D metabolism (100).

Catabolism of vitamin D begins with the 24-hydroxylation of either 25D or 1,25D to produce the inactive metabolites 24,25-dihydroxyvitamin D₃ and 1α,24,25-dihydroxyvitamin D₃, respectively, for excretion. These reactions are catalyzed by 25-hydroxyvitamin D-24-hydroxylase (CYP24A1), an enzyme primarily expressed in the kidney but also distributed in other tissues including the intestine and bone. CYP24A1 is also transcriptionally regulated by 1,25D. Essentially, increasing concentrations of 1,25D stimulate its own catabolism (101, 102).

Molecular Action

Circulating concentrations of 25D are reflective of an individual's vitamin D status. Concentrations vary depending on dietary intake and sunlight exposure (103, 104). Synthesis of 1,25D, on the contrary, is a tightly regulated process. The classical, hormonal actions of 1,25D related to mineral metabolism and skeletal health are well-established (Figure 1). Concentrations of 1,25D are influenced by parathyroid hormone (PTH), circulating calcium and phosphorous concentrations as well as fibroblast growth factor 23 (FGF-23) (97). Expression of CYP27B1, responsible for catalyzing 25D to 1,25D, is stimulated by PTH, whose primary function is to respond to a reduction in plasma calcium concentrations and act upon bone to stimulate calcium release, enhance active reabsorption of calcium from the kidney, and stimulate production of 1,25D, which in turn will increase absorption of calcium by the intestine (101). As plasma concentrations of 1,25D increase, a negative feedback loop suppresses CYP27B1 expression. FGF-23 concentrations increase, suppressing CYP27B1 and inducing CYP24A1, ultimately reducing vitamin D activation and subsequently decreasing calcium absorption (97, 101, 105, 106).

The biological responses to the 1,25D hormone are mediated by VDR, a DNA-binding transcription factor that activates a signal transduction complex, which includes the 1,25D-liganded VDR and retinoid X receptor (RXR). After 1,25D and VDR bind, the complex translocates from the cytosol to the nucleus where it forms a heterodimer with RXR. Together, the VDR-RXR complex binds to vitamin D responsive elements (VDREs) located in specific sequences near promoters and recruits coregulatory complexes to regulate transcription (97, 107, 108). The two primary functional units of VDR are the N-terminal zinc finger DNA-binding domain and the C-terminal ligand-binding domain. The presence of the 1,25D-RXR ligand results in a dramatic conformational change at the C-terminus of

VDR. Upon heterodimerization, TATA binding protein associated factors and D-receptor interacting proteins (DRIP) are recruited to the transcription site. Coupled together with RNA polymerase II, in addition to other co-activators, transcription is initiated (107). Furthermore, the conformational change induced by the 1,25D-RXR-VDR ligand has the added effect of converting VDR into a more efficient substrate for one or multiple serine protein kinases, potentiating transcriptional activity of the VDR-RXR heterodimer, said to be enhanced by interactions with, DRIP25, a coactivator (109, 110). VDR is also responsible for the down-regulation of transcription in a variety of genes. This down-regulation is accomplished when the VDR-RXR complex docks on a negative VDRE, binds a corepressor, and induces histone deacetylation and demethylation which alters the architecture of chromatin near the target gene (111). Additionally, it has also been demonstrated that VDR, when associated with the plasma membrane, has the potential to facilitate non-genomic actions by activating a variety of signal transduction pathways that can include kinases, phosphatases or ion channels (107, 111).

With respect to VDRE, the sequence of VDRE has been shown to strongly influence the level of protein binding (108). In other words, different sequences of VDRE promote distinctive conformations in the VDR-RXR complex, ultimately promoting heterodimer associations with specific comodulators and permitting differential actions in a variety of tissues. While majority of VDREs occur as one copy in the proximal promotor of vitamin D-regulated genes, it is now known that VDREs can exist in at least two regions of the gene (112-119). Genes that possess multiple VDREs require VDR-RXR docking sites for maximal induction by 1,25D and these individual VDREs have been shown to function together in recruiting coactivators for transactivation (107, 120-122). 1,25D-mediated gene expression

has been well-established in traditional vitamin D target tissues including kidney, bone, intestine, parathyroid, skin and the hematopoietic system. Importantly, identification of VDRE-containing genes comprises an even wider variety of biological networks including, metabolism, immune function and cell proliferation, differentiation, migration and death, demonstrating that vitamin D is involved in several of the most fundamental processes in life (107).

Status

Among the research and medical communities, it is agreed upon that the gold standard measurement of vitamin D status for an individual is serum 25D concentrations. A key area of debate among scientists and medical personnel relates to definitions of vitamin D status: deficiency, insufficiency, sufficiency and toxicity (123). In 2011, after extensive review of the literature, the Institute of Medicine (IOM) concluded that for maximum bone health, concentrations of 25D at 20 ng/mL and above was adequate (Table 1) (124); however, this recommendation was met with great criticism from the Endocrine Society. Their expert panel concluded that to guarantee bone health, blood concentrations of 25D of at least 30 ng/mL is required for the adult population (Table 1) (125). It is important to consider that the IOM guidelines are based upon the general population, while the Endocrine Society has taken into consideration individuals at risk for vitamin D deficiency (123, 126). Regardless, the existing controversy continues to generate confusion for clinicians, scientists, and the public. A number of factors contribute to vitamin D status including exposure to sunlight, skin pigmentation, and dietary intake (124). The Recommended Daily Allowance (RDA) for vitamin D is 600 IU/d with 4000 IU/d set as the Upper Tolerable Limit.

Table 1. Vitamin D status based on 25D concentrations

Vitamin D Status	Institutes of Medicine		Endocrine Society	
	ng/mL	nmol/L	ng/mL	nmol/L
Deficient	< 12	<30	<20	<50
Insufficient	12-20	30-50	21-29	53-73
Sufficient	>20	>50	30-100	75
Toxic	>50	>125	—	—

These values were established by the IOM to meet the skeletal health needs of healthy populations because poor vitamin D status can lead to rickets, osteomalacia and decreased bone mineral density. To achieve a serum 25D concentration of 20 ng/mL (50 nmol/L), 600 IU/d is said to be sufficient; however, whether it is an adequate amount to provide all the potential non-skeletal health benefits associated with vitamin D is debated (124). Evidence suggests that poor vitamin D status increases the risk for chronic diseases including obesity (127, 128), type 1 (129-131) or 2 (132-134) diabetes, chronic kidney disease (135, 136) and gastrointestinal disorders (137, 138). An intake of 2000-5000 IUs/ d, 5 times the RDA, has been recommended to achieve the IOM's optimal concentration of 20 ng/mL to support overall health (139-141). Several studies have evaluated the efficacy of high dose vitamin D on health outcomes. In one study designed to evaluate the effect of vitamin D3 supplementation in patients with bone metastases from breast cancer, the investigators concluded that 10,000 IU/d was a safe dose, though no significant changes in bone resorption was identified (142). Another study assessing 10,000 IU daily for 4 weeks in subjects with vitamin D deficiency and impaired fasting glucose, also found the dose to be harmless and not associated with undesirable side effects (143). Furthermore, a study in obese children demonstrated that a weekly dose of 25,000 IU vitamin D3 was well-tolerated and effective at improving the vitamin D status of participants who, at baseline, had insufficient and deficient

circulating concentrations (144). To that end, there remains a need for large-scale randomized clinical trials (RCT) designed to define the role of vitamin D supplementation in outcomes related to extraskeletal health (145). One such RCT is nearing completion in June 2018. The Vitamin D and Omega-3 Trial (VITAL) is a 2×2 factorial, double-blind, randomized, placebo-controlled trial investigating the benefits and risks of daily supplementation for 5 years with 2000 IU/d vitamin D3 and marine omega-3 fatty acids (Omacor® fish oil, 1 g/d) in the primary prevention of cancer and CVD in 25,871 U.S. men and women over the age of 55 (146). To date, VITAL is the largest randomized trial of vitamin D in the country and, globally, is the only large trial with racial and ethnic diversity, and thus has the potential to impact future changes to the dietary reference intakes for vitamin D (146).

While vitamin D toxicity is difficult to achieve from a combination of routine endogenous and exogenous sources, incidences of toxicity have been reported but are most often under accidental circumstances, e.g., prolonged, excess supplementation (124). The hallmark of vitamin D intoxication is hypercalcemia due to increased bone resorption and a subsequent rise in circulating calcium concentrations. Hypercalcemia can be life threatening if left untreated, leading to the deposition of calcium salts in soft tissues and organ dysfunction (124). There is a subset of the population that is at risk for vitamin D toxicity at normal serum 25D concentrations. Those with diseases that compromise the negative feedback on CYP27B1 in the kidney are more sensitive to developing toxicity. Individuals with chronic inflammatory diseases including tuberculosis, and sarcoidosis are also more susceptible to hypervitaminosis D because overproduction of extra-renal 1,25D by immune cells of the alveoli escapes normal feedback control by PTH causing hypercalcemia (147).

Additionally, mutations to CYP24A1, the enzyme responsible for the catabolism of 1,25D, results in increased circulating 1,25D. Individuals typically present to the hospital with symptoms of hypercalcemia, hypercalciuria and/or nephrolithiasis before a mutation is identified (148).

Vitamin D deficiency has been reported to affect an estimated 30% of the U.S. population (149). Pregnant and lactating women, the elderly, those with increased skin melanin pigmentation, and children and adults with insufficient sun exposure are at especially high risk for developing vitamin D deficiency (125, 150-152). In infants and children, severe vitamin D deficiency manifests into rickets, which is characterized by a failure of the cartilage to mature and mineralize into bone. The effects are evident at the wrists, ankles and knees, all of which become enlarged. Furthermore, as weight-bearing activity begins, the long bones of the legs begin to bow, and knees knock. In adults, deprivation of vitamin D leads to osteomalacia, which is characterized by defects in bone mineralization caused by alterations in calcium and phosphorous absorption and excretion (153). Specifically, 1,25D-mediated calcium absorption decreases. The decline in serum calcium concentrations trigger increased secretion of PTH. PTH promotes bone resorption and increased urinary excretion of phosphorous. Without adequate concentrations of serum calcium and phosphorous, the mineralization of bones under the direction of calcitonin cannot occur. Calcitonin is produced in the thyroid gland, is stimulated by increased calcium concentrations and facilitates the deposition of calcium and phosphorous in bone (154).

Vitamin D and Chronic Diseases

With the discovery of VDR and the presence of 1,25D activity in a number of tissues, accumulating evidence from observational data and small clinical trials have demonstrated an

association between vitamin D deficiency and a variety of chronic diseases (150). In studies of cancer cells and in tumors of animal models, vitamin D was found to be involved in a variety of physiologic processes that may slow or prevent malignancy, including promoting cellular differentiation, decreasing cancer cell growth, inducing apoptosis and reducing angiogenesis (155-158). A variety of molecular effects of vitamin D and the cardiovascular system have also been described (159). For example, mice with a systemic knockout of either VDR or CYP27B1 exhibit myocardial hypertrophy with overexpression of the renin-angiotensin-aldosterone system, hypertension, and advanced atherosclerosis (160). Vitamin D may also possess antihypertensive properties by suppressing the renin-angiotensin-aldosterone system (161, 162). Studies also suggest that vitamin D can protect against atherosclerosis and may even promote endothelial repair in vascular smooth muscle cells (163-165). Finally, because vitamin D is involved in immune modulation, a number of studies highlight the potential role for vitamin D in the pathogenesis of autoimmune diseases including multiple sclerosis, rheumatoid arthritis, T1D and Crohn's disease (100, 166-169). The effect of vitamin D on the innate immune system is said to be primarily through toll-like receptors, while its effect on the adaptive immune system is through T-cell differentiation, specifically type 17 T helper cell which is overactivated in autoimmune diseases and leads to inappropriate production of pro-inflammatory cytokines (170, 171). To date, however, the available data are not comprehensive enough to establish vitamin D recommendations for the prevention or treatment of chronic diseases. To fully understand the effects of vitamin D on chronic disease outcomes, rigorous, large-scale randomized clinical trials are necessary (150).

Diabetes

The potential therapeutic effects of supplemental vitamin D in the prevention or treatment of diabetes remains the subject of debate. Epidemiological and association studies suggest a correlation between vitamin D deficiency and an increased prevalence of T1D and T2D (172). Data from cross-sectional studies indicate that lower serum 25D status is associated with impaired insulin sensitivity in T2D (127, 173-176). Furthermore, it has also been demonstrated that overweight and obese individuals are more susceptible to vitamin D insufficiency or deficiency, which enhances their risk for T2D due to the role vitamin D may play in the maintenance of glucose homeostasis (177-180). In a study that investigated whether low circulating 25D concentrations are associated with impaired insulin function in individuals with prediabetes, it was determined that those with a combination of prediabetes and hypovitaminosis D were more insulin resistant and exhibited impaired β -cell function, compared to the group with normal fasting blood glucose, suggesting that vitamin D may play a role in regulating insulin and glucose homeostasis (173).

A number of observational studies have reported an improvement in vitamin D status, greater insulin sensitivity, and decreased fasting blood glucose following intervention with a vitamin D supplement (181-183). Specifically, Yousefi and colleagues reported a significant decrease in HbA1c in T2D patients who received 4000 IU vitamin D3 for 5 months compared to diabetic patients who received a placebo (181). Similarly, another study assessing the intervention of weekly 50,000 IU vitamin D3, for 8 weeks, in T2D participants, showed significant improvements in fasting blood glucose, insulin and in HOMA-IR. A recent systematic review and meta-analysis of RCTs, found that short-term vitamin D supplementation had a positive effect on fasting blood glucose in T2D patients specifically with poorly controlled diabetes (183). These trends have also been reported in cases of T1D

(184, 185). A prospective study evaluated the effect of 4000 IUs vitamin D₃, daily for 12 weeks in T1D participants who also had vitamin D deficiency. Patients that achieved lower HbA_{1c} also had higher serum 25D concentrations compared to T1D participants whose vitamin D status did not improve (186).

The genomic action of 1,25D and the presence of VDR in the pancreatic β -cell and their role in insulin secretion may provide a mechanistic link to the pathogenesis of diabetes (187, 188). In a study of VDR-mutant mice, impaired insulin secretory capacity was exhibited, and mRNA expression of insulin was lower compared to wild-type mice (189). Similarly, another study demonstrated that vitamin D deficient rats exhibited improved insulin secretion upon repletion with 1,25D (190). Furthermore, in a model of T1D, treatment of 1,25D prevented the onset of diabetes in non-obese diabetic (NOD) mice by suppressing islet expression of pro-inflammatory cytokines and a reduction in insulinitis; however, late intervention treatment of 1,25D was unsuccessful in preventing diabetes incidence in NOD mice (191), demonstrating that the beneficial effects of 1,25D are restricted in the presence of immune cell infiltration. Action of 1,25D on glucose homeostasis in peripheral tissues was exemplified in a study with glucose-treated 3T3L1 adipocytes. Treatment of 1,25D upregulated GLUT4 expression and its translocation to the cell surface, leading to an increase in glucose uptake (192).

Despite the many described benefits of vitamin D in the prevention and management of diabetes, several studies argue the contrary (193-196). A recently published double-blind, randomized, placebo-controlled study determined that intervention with 4000 IU vitamin D₃ per day for 11 months did not improve HbA_{1c} or insulin secretion rate, measured by C-peptide concentration, in T2D participants with well-controlled disease (196). Similarly,

intervention with weekly 30,000 IU (193) and 28,000 IU (194) reported no effect on β -cell function, glucose homeostasis or insulin sensitivity in participants with prediabetes and diet-controlled T2D. In another study, daily intake of 0.25 μ g 1,25D for 9 months, while determined safe, did not improve β -cell function in participants with newly diagnosed T1D (195). Overall, the results of current randomized clinical trials on the effect of vitamin D in patients with impaired glucose tolerance or type 2 diabetes are inconsistent (183, 197, 198). In the future, large-scale trials with a long-term intervention period will be critical to understanding the glycemic effects of vitamin D treatment in the prevention and management of T1D and T2D.

IBD

Vitamin D deficiency has long been recognized as an environmental risk factor for CD. This finding was initially correlated with the incidence of bone disease in individuals with CD (67). As reviewed by Del Pinto et al. (137), emerging data suggest that low serum 25D status has a significant role in disease activity in IBD. Moreover, diarrhea, malabsorption, and GI bleed are common features of IBD; thus, nutritional deficiencies are common in individuals with UC and CD (199). While the role of vitamin D signaling in the gut has not been fully elucidated, plausible mechanisms related to immunological action have been demonstrated. In a healthy individual, sufficient circulating 25D concentrations assist gut epithelial barrier integrity. When the barrier is penetrated by luminal microbiota, activation of toll like receptors (TLRs) on antigen presenting cells (APCs) induces intracrine vitamin D signaling in APCs to contain the microbes (200, 201). Additionally, target genes for 1,25D include proteins involved in the formation of tight junctions such as occludins and

claudins, as well as proteins involved in autophagy and the expression of antimicrobial peptides (AMPs), which together preserve intestinal immune homeostasis (200). Therefore, under vitamin D deficient conditions, when flux through CYP27B1 is diminished, APCs are not able to respond as efficiently to bacterial insult. Consequently, there may be a reduction in tight junction formation, causing an increase in bacterial ligands to TLRs and NOD2, ultimately leading to initiation of the host immune response (200). In a study comparing VDR knockout mice with wildtype mice, researchers identified higher expression of proteins involved in cell proliferation and migration and stress response in VDR knockout mice, implicating a role for vitamin D in maintenance of these functions (202). Similarly, Wu et al. (203) determined that ATG16L1, an autophagy gene, was downregulated in VDR knockout mice, and these mice were also more susceptible to DSS-induced colitis compared to wildtype mice. Moreover, VDR/IL-10 double knockout mice develop severe IBD involving the entire small intestine and colon and exhibit significant changes in colonic tissue and inflammation compared to single VDR and IL-10 knockout mice, which may indicate that VDR expression is required to manage intestinal inflammation (204).

In humans with IBD, hypovitaminosis D is associated with lower quality of life, higher disease activity scores and increased morbidity (205, 206). In a small, randomized, double-blind placebo-controlled trial, Jørgensen and colleagues (207) determined that daily 1200 IU vitamin D3 for 12 months increased serum 25D concentrations and was associated with a reduced rate of relapse in participants with CD. In another study carried out among patients with IBD, weekly 50,000 IU vitamin D3 for 3 months resulted in increased circulating 25D and diminished serum TNF- α concentration, which suggests an improvement in the immune response (208). Of note, the difference in TNF- α concentration from baseline

to post intervention was reported to be statistically insignificant ($P = 0.07$) (208). The investigators propose a longer intervention period to yield a more robust immune response to supplemental vitamin D3. To date, however, there remains no standardized protocol for supplemental vitamin D recommendations to manage immunomodulatory-mediated extraskeletal complications of IBD (209). With respect to risk of disease, results from women enrolled in the Nurses' Health Study demonstrated that higher serum 25D concentrations significantly reduced the risk for incident CD but not UC (210). The relationship between sun exposure and risk of developing CD or UC has been investigated with respect to geographical location and seasonal variation. Studies indicate that lower UVB exposure is associated with a higher risk of CD, but not UC (211-213).

Though much advancement has been made in understanding the mechanism of vitamin D action and its effect on mucosal and systemic immune system, including intestinal inflammation, further studies are needed to identify the optimal concentration of circulating 25D for immunomodulatory effects in IBD and establish supplement recommendations for therapeutic management (209).

Dietary Sources

As described previously, vitamin D needs are met by most individuals through exposure to sunlight. Alternatively, vitamin D is acquired through the diet. The abundance of a vitamin D-rich foods in the market is insufficient because vitamin D occurs naturally in very few foods. Table 2 (214) contains a list of foods, their content of vitamin D and the percent daily value for reference. Among the best sources are fatty fish and supplemental fish liver oils, which include tuna salmon and mackerel (215). Vitamin D3 and 25D are also found in small amounts in beef liver, egg yolks and some cheeses. Lastly, certain mushrooms

contain variable amounts of vitamin D₂, depending on their UVB exposure (215). In America, as well as other countries (216-218), the majority of vitamin D in foods is provided by fortification (219). The milk fortification program was implemented in the U.S. to combat rickets, which was a major health concern among children in the 1930's (124). Today, nearly all of the country's milk supply is fortified with 100 IU per 8 fl oz., though this program has not led to fortification of other dairy products (124). Specific brands of orange juice, margarine, yogurt and ready-to-eat breakfast cereals are other foods that contain added vitamin D. Additionally, there is a mandate in the U.S. to fortify infant formula with approximately 40-100 IU per 100 kcal (124).

Animal-based foods, including beef, poultry and eggs, are known to contain varying amounts of 25D, the metabolized form of vitamin D that represents an individual's status (214). In the U.S., the amount of 25D in foods has not been analyzed and thus, is not included when reporting the total vitamin D content of foods. Adding to the complexity is the fact that there is no standard reference for measuring 25D in food matrices (220, 221). Recent evidence suggests that 25D is approximately five times more potent than vitamin D₃ in raising circulating 25D concentrations (45, 221). For this reason, intake of vitamin D across the U.S. and the contribution of 25D to the vitamin D status of Americans may be underestimated (221). Advancements are underway to identify validated methodologies to determine amounts of vitamin D and 25D in foods and supplements. These endeavors have the potential to address the discrepancies between reported serum 25D concentrations and vitamin D intake and contribute to public health policy decisions regarding vitamin D requirements (222).

Table 2. Food Sources of Vitamin D*

Food	IUs per serving	% Daily Value
Cod liver oil, 1 tbsp	1360	340
Swordfish, cooked, 3 oz	566	142
Salmon (sockeye), cooked, 3 oz	447	112
Tuna fish, canned in water, drained, 3 oz	154	39
Orange juice fortified with vitamin D, 1C	137	34
Milk, nonfat, reduced fat, and whole, vitamin D-fortified, 8 fl oz	120	30
Yogurt, fortified with 20% of the DV for vitamin D, 6 oz	80	20
Margarine, fortified, 1 tbsp	60	15
Sardines, canned in oil, drained, 2 sardines	46	12
Liver, beef, cooked, 3 oz	42	11
Egg, 1 large	41	10
Ready-to-eat cereal, fortified with 10% of the DV for vitamin D, 0.75-1C	40	10
Cheese, Swiss, 1 oz	6	2

*Adapted from the National Institutes of Health Office of Dietary Supplements (214)

Dietary Whole Eggs

In the U.S., egg consumption per capita was estimated to be 275 in 2017, equating to less than 1 egg per day per person (223). There are no specific guidelines for daily or weekly egg consumption; however, studies show that 1 egg per day is not detrimental to health (224, 225). As part of a healthy eating pattern, it is suggested to incorporate whole eggs into a balanced diet containing a variety of fresh produce, whole grains and lean meats (226). Fundamentally, eggs possess culinary versatility, low economic cost, and no religious-based restrictions, which make them an appealing food to a variety of populations around the world (227, 228).

Egg Nutrition

It is well established that eggs contain a variety of nutrients, particularly compared to other animal products (226). They have long been promoted for their high-quality protein, possessing an amino acid profile that is similar to beef but has greater bioavailability, as well as for their high nutrient density-to-energy ratio (226, 227, 229). The caloric content of 1 egg equates to 3.6% of total calories, relative to a 2000 calorie diet, while at the same time providing a number of other nutrients (Table 3) in excess including folate, riboflavin, choline, vitamin B12, vitamin A and vitamin D (230).

Choline can be acquired via de novo biosynthesis, through methylation of phosphatidylethanolamine, which alone is not sufficient to meet the daily requirement and must be met via dietary intake (231). Eggs not only contain a high concentration of choline, but they are one of few dietary sources of choline. As a component of egg lecithin, choline possesses a number of important physiologic functions, including the synthesis of

phospholipids such as phosphatidylcholine, a predominant component of all biological membranes (227, 229, 231). Additionally, choline functions in methyl group metabolism, neurotransmitter synthesis, and lipid transport (227, 231).

One underappreciated group of nutrients also found in eggs are carotenoids, the natural pigment of egg yolks giving it its yellow-orange color. Lutein and zeaxanthin are the primary carotenoids found in egg (227). These carotenoids accumulate in the macular region of the retina and have been implicated in the prevention of age-related macular degeneration (232). Because lutein and zeaxanthin are not synthesized endogenously, their circulating concentrations are dependent on dietary intake. Furthermore, the food matrix in which carotenoids are found may impact their bioavailability. In egg yolks, lutein and zeaxanthin are associated with the lipid matrix, making them highly bioavailable (227, 229, 232).

Egg yolks are also a rich source of vitamin D owing to the efficient transfer of vitamin D, by the hen from the feed into the yolk (233). As described earlier, egg contains both vitamin D₃ and 25D. It has been reported that eggs contain higher concentrations of 25D compared to other animal-based foods, which is significant when considering the potency of 25D (221, 234). It is estimated that 1 egg (50 g) contains 0.325 µg, or 13 IU 25D (221). After applying a potency factor of 5, the value increases to 65 IU 25D, which meets 11% of the RDA for vitamin D. Because eggs are naturally a good source of both vitamin D₃ and 25D, they are an ideal candidate for fortification. Browning et al. (234) demonstrated that supplementation of hen feed with varying amounts of vitamin D₃ and 25D resulted in significant increases in the egg yolk concentrations of vitamin D₃ and 25D, suggesting that egg consumption has the potential to contribute to the RDA requirement of vitamin D (234).

Table 3. Nutrient Composition of Eggs*

Nutrient	1 large, cooked, poached (50 g)	Vitamin and Mineral % RDA
Energy (kcal)	72	—
Protein (g)	6.25	—
Total lipid (g)	4.74	—
Saturated fat (g)	1.556	—
Monounsaturated fat (g)	1.821	—
Polyunsaturated fat (g)	0.952	—
Cholesterol (mg)	185	—
Calcium (mg)	28	3
Iron (mg)	0.88	men 11 women 5
Magnesium (mg)	6	2
Phosphorus (mg)	98	17
Sodium (mg)	148	—
Zinc (mg)	0.65	7
Thiamin (mg)	0.016	1.3
Riboflavin (mg)	0.194	16
Niacin (mg)	0.032	0.2
Vitamin B ₆ (mg)	0.072	6
Folate, DFE** (μg)	18	5
Choline (mg)	117	23
Vitamin B ₁₂ (μg)	0.35	15
Vitamin A, RAE** (μg)	80	10
Vitamin D (IU)	41	7
Vitamin K (μg)	0.1	0.1

* USDA National Nutrient Database (230)

**DFE = dietary folate equivalent, RAE= retinol activity equivalents

Egg consumption and health

Satiety

The relationship between dietary protein and satiety is well defined, as dietary protein enhances satiety and reduce food intake beyond the necessary isocaloric needs of the individual (226). Importantly, there is evidence to suggest that the high protein content of eggs may contribute to post prandial satiety (235-238). A recent study in Sprague Dawley

rats compared the influence of protein level and source on satiety. The investigators determined that egg white protein provided at 35% of total calories, was superior to wheat gluten (35% protein) and a basal diet (20% protein) at increasing satiety (238). On the contrary, in a study carried out in healthy men, isocaloric, macronutrient-balanced, fiber-matched meals based on vegetable protein or animal protein (including eggs) had similar effects on hunger, satiety, and fullness based on measurements using visual analogue scales (239). Because the test meals in this study contained a combination of whole foods, the results may be more reflective of normal human consumption. Additionally, a number of studies have investigated the effect of egg consumption on satiety with respect to specific meal time. Some research has shown that, when provided at breakfast, eggs enhance satiety (235, 237) and reduce short-term energy intake (235, 240), while others found no impact on appetite and food intake after egg intake at breakfast (241). Similarly, an egg omelet at lunch elicited a significantly stronger satiating effect in participants compared to a potato-based meal, though energy intake was similar between groups 4 hours post meal intervention (236).

Allergy

Egg allergy is the second most common food allergy, second to cow's milk, affecting 1-2% of infants and children worldwide (242). The four primary allergenic proteins are found in the egg white and consist of ovomucoid, ovalbumin, ovotransferrin and lysozyme. Egg yolk allergens have also been identified, though hypersensitivity to these proteins are less common. They include chicken serum albumin and YGB42 (242, 243). Allergy to egg is considered a type 1 hypersensitivity reaction owing to the rapid inflammatory response characterized by overproduction of immunoglobulin E (IgE) and may manifest into itching, atopic dermatitis, bronchial asthma, vomiting, rhinitis, conjunctivitis, laryngeal edema,

chronic urticaria, or anaphylaxis (243). The relationship of egg consumption and an immune response is complex. There is no cure or treatment that provides long-term remission for egg allergy. Strict avoidance of eggs and egg-containing products is the most effective way to avoid an immune reaction (244). It is common, however, for children to outgrow an egg allergy. The reported rate of resolution in the US ranges from 12-68%, depending on the age of resolution (245). Elucidating the mechanisms involved in egg allergy remains complex. Advancements in genetics, epigenetics, environmental factors and the gut microbiome show promise in understanding the triggers and subsequently the treatment of egg allergy (244).

Cardiometabolic Disease

The relationship between serum cholesterol and heart disease was first identified in the Framingham Heart Study, which hypothesized that dietary cholesterol, via circulating lipids, contributed to heart disease risk (246, 247). In the U.S., the estimated cholesterol intake averages 200-350 mg/ day. With approximately 200 mg of cholesterol in a single large egg (230), eggs make up a significant source of dietary cholesterol in the American diet (248, 249). Initial observational studies established a link between dietary cholesterol and CVD risk; however, these early studies did not account for confounding variables including dietary and lifestyle habits (250, 251). As a result, egg consumption has been the focus of countless studies, and for decades limited egg consumption has been recommended to reduce CVD risk, particularly in people with T2D (252). Importantly, revisions to the Dietary Guidelines for Americans in 2015 no longer include recommendations to limit intake of dietary cholesterol, a decision based on the growing body of literature demonstrating a lack of association between cholesterol intake and adverse health risks in the general population (253). Consistent with this message, organizations around the world, including the American

Heart Association, The British Heart Foundation, The National Heart Foundation of Australia and the Danish Heart Association, are loosening restrictions on egg consumption (252). Despite this international benchmark, the debate on egg consumption and CVD risk continues.

An important fact to consider are individual serum cholesterol responses and adaptations to cholesterol intake. The majority of cholesterol in the body is produced endogenously (30,31). Approximately 25% of serum cholesterol is derived from the diet. To provide perspective on those values, Blesso and Fernandez (254) state that the average 70 kg adult synthesizes an estimated 850 mg cholesterol/day. If that adult consumed 400 mg/d of dietary cholesterol, the amount found in 2 large eggs, and absorbed 60% (255), that amounts to 240 mg from the diet of 1090 mg total cholesterol in the body, or 22% from diet (254). To add to the complexity, cholesterol balance is affected by synthesis rates of cholesterol and bile acids, and excretion rate from the body. Studies on sterol balance in high cholesterol diets demonstrate feedback inhibition of cholesterol biosynthesis and increased excretion of bile acids (256). Sterol regulatory element-binding protein-2 (SREBP-2) tightly regulates cellular cholesterol biosynthesis. When cellular cholesterol is decreased, activity of SREBP-2 increases to upregulate gene expression of proteins involved in cholesterol biosynthesis, such as 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase (257). Under conditions when cellular cholesterol biosynthesis is increased, both cholesterol biosynthesis and lipoprotein uptake are reduced via feedback inhibition. Here, SREBP-2 gene expression is reduced, and post-translational degradation of HMG-CoA reductase is enhanced (257). Feeding studies used to create predictive equations to estimate the response of dietary cholesterol intake on serum cholesterol estimate a 2.2-2.5 mg/dL change in serum cholesterol per 100 mg dietary

cholesterol (258). Thus, the majority of individuals respond marginally to dietary cholesterol due to feedback regulation of cholesterol stores. Importantly, additional genetic and metabolic factors are key players in this response. A number of clinical trials carried out in children (259), men (260), women (261) and older adults (262) have demonstrated variable responses to an additional 500-650 mg of egg-derived dietary cholesterol for a minimum of 4 weeks. The terms for this variation are “hyper- and hypo-responders” (263). Those who do not respond or respond with a slight increase in serum cholesterol when consuming a large amount of dietary cholesterol are hypo-responders, representing two-thirds of the population. Hypo-responders have the ability to compensate for the increase in cholesterol intake by decreasing biosynthesis, absorption, and increasing excretion (263, 264). On the contrary, the other third of the population are hyper-responders, in that they respond with large increases in serum cholesterol to high dietary cholesterol intake. This fact likely accounts for the heterogeneity in results across studies and meta-analyses on the relationship between egg consumption and CVD risk (263, 264).

A meta-analysis conducted by Shin et al. (265) determined that egg consumption was not associated with the risk of CVD or mortality in the general population (265). Similarly, in a cohort of healthy adults, Alexander et al. (266) found a 12% decrease in stroke risk and no association of coronary heart disease (CHD) with 1 egg per day compared to less than 2 eggs per week (266). A dose-response meta-analysis of prospective cohort studies also found no evidence of an association between egg consumption and risk of stroke or CHD (267). In another observational study, no association was found between egg consumption and risk of myocardial infarction and stroke; however, more than 1 egg per day was linked to an increased risk of heart failure in men (268). Choi and colleagues (269) examined the

association between egg intake and coronary artery calcium content in healthy South Korean subjects (269). In a multi-variable adjusted model, they identified a higher prevalence of coronary artery calcium in those who consumed greater than 7 eggs per week compared to subjects who consumed less than 1 egg per week. It was concluded that egg consumption is positively associated with atherosclerosis owing to the cholesterol content of the eggs. Of note, the subjects in which these associations were highest also presented with higher body mass index (BMI) and lower vegetable intake (269).

Interestingly, in diabetics, the association between egg intake and CVD appears to be more consistent (224, 267, 270, 271), though not always observed (272-274). In a meta-analysis, Li et al. reviewed 14 studies and identified a dose-dependent relationship between egg consumption and risk of CVD and T2D (275). The investigators determined that an increment of 4 eggs per week increased the relative risk for CVD by 1.06 (95% CI: 1.03–1.10), and T2D by 1.29 (95% CI: 1.21–1.37) (275). In another meta-analysis, restricted to studies in the U.S., consumption of 3 or more eggs per week increased risk of T2D (276). In the meta-analysis by Shin et al. (265), 4 cohorts included subjects with T2D. Those with T2D who consumed more than 1 egg per day were 1.69 times as likely to develop CVD than diabetics who never consumed eggs or ate less than once per week (265). Controlled studies, carried out in participants at high risk of T2D or with established T2D, demonstrate favorable effects or no adverse effects of high egg consumption on cardiometabolic risk factors or glycemic control (277-283). Pearce et al. demonstrated that a high-protein low-calorie diet, high in cholesterol derived from eggs, improved glycemic control, circulating lipids and blood pressure in participants with T2D. Similarly, the DIABEGG trial (282) investigated whether a high-egg diet (2 eggs/day for 6 days per week) compared to a low-egg diet (<2

eggs per week) affected circulating lipid profiles in prediabetics and subjects with T2D who were also overweight or obese, as part of a weight maintenance study. There were no adverse effects on the lipid profile of T2D subjects, suggesting eggs may be included as part of a balanced diet in T2D (282). In a recent follow-up study, the investigators assessed the effects of a high-egg versus low-egg diet as part of a 3-month weight-loss period and 6-month follow-up period in participants with prediabetes and T2D and an average BMI of 33.5 kg/m² (284). Results corresponded to their previous study. Those with prediabetes or T2D who consumed the high-egg weight loss diet exhibited no adverse changes in cardiometabolic markers compared to the low-egg diet group. This finding supports the incorporation of egg intake as part of healthy diet recommendations to induce weight loss in T2D (284).

Some studies have demonstrated poor cholesterol absorption in obese and insulin-resistant individuals compared to those who are lean (285-287). Thus, the hypothesis that the phosphatidylcholine content of eggs, as oppose to the cholesterol, may contribute to the increased susceptibility of cardiometabolic disease in high egg consumers, aligns with the observational studies that demonstrate a consistent link between egg consumption and CVD (288). Phosphatidylcholine is metabolized by intestinal bacteria, producing trimethylamine N-oxide (TMAO), which has been shown to promote atherosclerosis in a hyperlipidemic mouse model (289) and associated with increased adverse cardiovascular events in humans (290). However, in healthy young adults, 2-3 eggs per day was not associated with increased circulating concentrations of TMAO (237, 291). Moreover, postprandial plasma TMAO concentrations were significantly lower in healthy men following egg consumption compared to fish intake, a direct source of dietary TMAO (292). Overall, the dietary phospholipid

contribution to TMAO concentrations and subsequent CVD risk is multifaceted and requires further studies (293, 294).

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CHAPTER 3. WHOLE EGG CONSUMPTION PREVENTS DIMINISHED SERUM 25-HYDROXYCHOLECALCIFEROL CONCENTRATIONS IN TYPE 2 DIABETIC RATS

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Abstract

Type 2 diabetes (T2D) is characterized by vitamin D deficiency owing to increased urinary loss of 25-hydroxycholecalciferol (25D). Whole eggs are a rich source of vitamin D, particularly 25D, the circulating form that reflects status. Zucker diabetic (type 2) fatty (ZDF) rats and their lean counterparts were fed casein- or whole egg-based diets for 8 weeks. Whole egg consumption attenuated both hyperglycemia and hypertriglyceridemia, as well as reduced weight gain in ZDF rats compared to casein-fed diabetic rats. Circulating 25D was lower in casein-fed ZDF rats compared to lean controls; however, ZDF rats fed whole egg exhibited the same circulating 25D concentration as casein-fed lean rats. These data suggest that dietary whole egg can attenuate metabolic anomalies, as well as maintain normal circulating 25D concentrations in T2D rats. This finding may support new dietary recommendations targeting vitamin D deficiency prevention in T2D.

Introduction

Millions of individuals in the US population suffer from type 2 diabetes (T2D) and in combination with obesity, represents an epidemic disease that is continuously on the rise (1). Current recommendations focus on two aspects of T2D and obesity, controlling diet and

increasing physical activity. Dietary recommendations focus primarily on caloric intake and the consumption of specific forms of carbohydrate as a means to control blood glucose concentrations and reduce body weight. Inadequately controlled or uncontrolled T2D has adverse health implications with respect to numerous complications, including diabetic nephropathy and cardiovascular disease (2, 3). There are a number of emerging fields relevant to T2D and its associated complications. In particular, it is now recognized that T2D is characterized by micronutrient imbalances, including vitamin D (4).

Cholecalciferol (vitamin D₃) can be produced endogenously in the skin via UVB irradiation-induced conversion of 7-dehydrocholesterol or obtained in the diet from naturally occurring or fortified foods. In the liver, 25-hydroxylase functions to hydroxylate cholecalciferol to form 25D. Other organs expressing 25-hydroxylase include the lungs, intestine and kidneys. Once generated, circulating 25D reflects an individual's vitamin D status, measured by serum or plasma 25D concentrations. Currently, optimal levels have been poorly defined, and recommendations are controversial. It has been proposed that as many as 70% of Americans may be insufficient with respect to vitamin D status, based on serum 25D concentrations (5).

Following hydroxylation in the liver, 25D bound to vitamin D binding protein (DBP) is released into the blood stream and taken up primarily by the kidney. In the proximal tubule, 25D-DBP is endocytosed from the renal filtrate via the proteins megalin and cubilin. Taken together with disabled-2 (Dab2), an intracellular adaptor protein, 25D-DBP may either be returned to the circulation or undergo hydroxylation by 25D-1- α -hydroxylase, resulting in the production of 1,25-dihydroxycholecalciferol (1,25D), commonly referred to as calcitriol and considered the most active form of vitamin D. Therefore, optimal concentrations of 25D and

1,25D are dependent on renal function. This is a significant concern for individuals with diabetic nephropathy, as the ability to maintain adequate circulating concentrations of 25D and 1,25D may be compromised. Furthermore, with a propensity for compromised vitamin D status, diabetics are at increased risk for developing vitamin D deficient-related illnesses, including bone disease, autoimmune disorders, and a variety of cancers (6).

Whole eggs are an excellent source of vitamin D, specifically in the yolk (7). Thus, their incorporation into the diet represents a viable means to maintain vitamin D balance as a function of T2D despite the urinary loss of 25D and DBP. In this present proof-of-principle study, our objective was to understand the relation between egg consumption and T2D, particularly with respect to vitamin D balance and diabetic complications. Moreover, the primary source of vitamin D found in eggs is 25D (8). The literature remains inconsistent regarding the relation between egg consumption, glycemic control, and the onset and/or progression of T2D. There are limited mechanistic studies that have examined the relation between egg consumption, T2D progression, and its related adverse characteristics (9). Using a rodent model is essential to ascertain a level of comprehensive understanding that is required to develop a translational approach for humans. Furthermore, the majority of pharmacological therapeutic protocols for treatment of T2D are not completely sufficient; thus, dietary recommendations are of high importance as an alternative approach.

Materials and Methods

Chemicals. ELISA kits were purchased from the following companies: creatinine, Cayman Chemical; triglyceride, BioAssay Systems; 25-hydroxyvitamin D, Immunodiagnostic Systems. All other chemicals were analytical grade.

Animals and diets. All animal studies and diets were approved by the Institutional Animal Care and Use Committee at Iowa State University and were performed according to the Iowa State University Laboratory Animal Resources Guidelines. Ten 8-week old male Zucker diabetic fatty (ZDF; fa/fa) rats and 10 male lean control ZDF (+/?) rats were purchased from Charles River Laboratories. Rats were housed in individual plastic cages in a temperature-controlled room with a 12-h light-dark cycle. All diet ingredients, with the exception of dried whole egg (Rose Acre Farms, Guthrie Center, IA), were purchased from Harlan Teklad (Madison, WI), as well as L-methionine and choline bitartrate (Sigma Aldrich, Milwaukee, WI). All rats were acclimated to a standard semi-purified diet (AIN93G) for 3 days. Rats were randomly assigned to one of two diet groups: a control casein-based diet, or the same diet containing whole egg in place of casein. Both diets provided dietary protein at 20% (w/w). Additional corn oil was added to the casein-based diet to achieve the same total lipid content as the whole egg-based diet. The vitamin mix (TD.94047) contributed 25 µg of vitamin D/ kg of diet. Dried whole egg provided an additional 12.6 µg vitamin D per kg of the experimental diet, which is ~50% greater than the level provided by the vitamin mix in the casein-based diet (Table 1). All diets and water were provided ad libitum. For diet preparation, dry ingredients were individually weighed and combined in a countertop 12-quart mixer (Hobart, Troy, OH). After mixing the dry ingredients, wet ingredients were blended in during the final mixing stage. Diets were stored at 4°C throughout the duration of the study and experimental diets were initiated on day 4, following the acclimation period.

Study design. Rats were maintained on their respective diets for 8 weeks, thereby reaching a sufficient age to ensure a diabetic state was achieved in the ZDF rats, as well as vitamin D

insufficiency 6. Prior to sacrifice on day 65, rats were housed in metabolic cages during which food was withheld, but water was provided ad libitum. After 12 h, urine was collected and stored at -80°C until analysis. Rats were anesthetized with a ketamine: xylazine cocktail (90:10 mg/kg body weight) via a single intraperitoneal injection and whole blood was collected via cardiac puncture. Liver, kidney, and abdominal fat were removed and weighed. Euthanasia was achieved by exsanguination.

Biochemical assessment. Blood glucose concentrations were measured at the time of sacrifice using a glucometer (Bayer Inc., Mississauga, Canada). Urinary creatinine concentrations were measured using a commercial colorimetric kit (Cayman Chemical, Ann Arbor, MI), as previously described (10). Plasma triglyceride concentrations were measured using a commercial colorimetric kit (BioAssay Systems, Hayward, CA). The assay uses a one-step combination of triglyceride hydrolysis, glycerol formation and dye reagent oxidation. Color intensity is measured at 570 nm and is proportional to triglyceride levels in the plasma. Plasma and urinary 25D were measured by using a commercial enzyme immunoassay kit (Immunodiagnostic Systems, Scottsdale, AZ) as previously described (6). Total 25D excreted in the urine was calculated and normalized to urinary creatinine.

Statistical analysis. All data were analyzed using SigmaPlot 9.0 (Systat Software Inc). Evaluation of statistically significant differences ($P < 0.05$) between group means was performed using a two-way ANOVA (genotype x diet) followed by Student-Newman-Keuls Method for multiple comparisons.

Results

Body and Tissue Weights. The cumulative weight gain in the 4 treatment groups over the 8-week experimental period is shown in Figure 1. Initially, ZDF rats fed the casein-based diet gained weight more rapidly than the other groups. There were no significant differences in weight gain patterns between the lean casein and lean whole egg groups. On d 29, the cumulative weight gain exhibited by the ZDF rats in the whole egg group began to plateau compared to the other 3 treatment groups whose weight continued to increase. At the conclusion of the study, ZDF rats fed the whole egg-based diet exhibited a 40% decrease in cumulative weight gain compared to the casein-fed ZDF rats, as well as both lean control groups ($P < 0.001$). Final body weight and tissue weights (g/100 g body weight) are presented in Table 2. Though no significant differences were observed in liver, kidney or adipose relative weights across diets within the ZDF or lean rats, there were notable differences in these relative tissue weights between genotypes. Regardless of diet, ZDF rats exhibited an 83, 36, and 267% increase in the relative weight of the liver, kidney and abdominal adipose tissue, respectively. Similar to the cumulative weight gain in Figure 1, the final body weight of ZDF rats fed the whole egg diet was 20% lower than ZDF rats fed a casein-based diet.

Blood Glucose and Plasma Triglycerides Concentrations. Comparisons of blood glucose and plasma triglyceride concentrations are shown in Figure 2. As expected, ZDF rats had an approximately 4-fold increase in blood glucose concentrations at the end of the 8-week experimental period compared to lean rats where both groups were fed the casein-based diet. There was no significant difference in blood glucose concentrations between lean rats fed a whole egg- or casein-based diet. However, the whole egg-based diet attenuated hyperglycemia by 49% in ZDF rats ($P = 0.008$). Similar to blood glucose concentrations, plasma triglyceride

concentrations were highest in ZDF rats fed the casein-based diet. No statistical significance was observed in plasma triglyceride concentrations between the two diets in lean rats. However, consumption of the whole egg-based diet resulted in a 52% decrease in plasma triglyceride concentrations in ZDF rats fed the egg-based diet compared to casein ($P < 0.001$).

Plasma 25D Concentrations. As expected, plasma 25D concentrations of ZDF rats fed the casein-based diet were 53% lower than lean rats fed the casein-based diet (Figure 3). However, diabetic rats fed the whole egg-based diet exhibited circulating 25D concentrations that were the same as lean controls fed the casein containing diet. Furthermore, circulating 25D concentrations in ZDF rats in the whole egg group were 148% higher than ZDF rats in the casein group ($P = 0.009$). Whole egg also resulted in a 2-fold increase in circulating 25D concentrations in lean rats ($P < 0.001$). As expected, the whole egg-based diet was without effect on diminished urinary creatinine concentrations nor the loss of 25D in the urine of ZDF rats (data not shown).

Discussion

The abundance of vitamin D-rich foods available in today's market are insufficient and do not meet the recommended daily requirement for healthy Americans. Furthermore, researchers have postulated that a large proportion of the population exhibits serum 25D concentrations that are substantially lower than what is required for reducing chronic disease risk (11-14). This proof-of-principle study clearly demonstrates that whole egg consumption results in increased plasma 25D concentrations, attenuated hyperglycemia and hypertriglyceridemia, as well as reduced weight gain in T2D rats. These findings suggest that dietary whole egg consumption is a valuable source of vitamin D, 25D in particular, for the

T2D phenotype characterized by vitamin D insufficiency. Because whole egg is a natural food source of 25D it represents an ideal dietary strategy to maintain vitamin D homeostasis in T2D, as well as reducing characteristic elevations of plasma glucose and triglycerides.

Though 25D is known to be present in eggs and other animal-based foods, there is no current standardized method to determine 25D concentrations. The amount reported is inconsistent and is not included in estimates of vitamin D intake in the U.S (8, 15, 16). A recent study by Taylor et al. (8) utilized the concept of a potency factor to better determine 25D activity in meat and eggs. The authors designated 5 as an appropriate potency factor for their calculations based on the strength of previous studies. It also represents the middle value within the range of reported potency factors. Ostensibly, when the vitamin D content of these foods was recalculated using a potency factor of 5, concentrations of 25D increased in microgram increments. Preliminary USDA data estimates $0.65 \pm 0.08 \mu\text{g}$ 25D/100 g whole egg. After the potency factor is applied the value increases to $3.25 \mu\text{g}$ 25D/100 g whole egg, or 130 IU (8). To put it in perspective for the consumer, a standard large egg of 50 g contains 65 IU of 25D, which provides 11% of the DRI (17).

The current literature regarding the impact of whole egg consumption for individuals with T2D is contradictory; thus, dietary recommendations for individuals with T2D and egg consumption are problematic. Although some studies concluded egg consumption increased T2D risk and related complications, others have not found such an association (18-20). A study by Pearce et al. (21) reported that egg consumption, along with other dietary modifications, improved glycemic and lipid profiles in individuals with T2D. Similarly, others have found diets containing whole eggs exhibit a positive impact on glucose tolerance and insulin sensitivity (9, 22). Here, we demonstrated that whole egg consumption resulted in

approximately a fifty percent reduction in hyperglycemia. A likely explanation for this could be a diet effect on body weight, with respect to the relationship between obesity and impaired glucose homeostasis. The same group of rats, with improved glucose concentrations, also gained significantly less weight than the other three groups. Because we did not monitor food intake, it is possible that ZDF rats consumed less of the whole egg-based diet; however, a number of arguments diminish the likelihood of this explanation. First, lean control and ZDF rats fed the casein-based diet, as well as lean rats fed the whole-egg based diet followed the same pattern in weight gain. This eliminates the argument that the whole egg content of the diet itself would result in a decrease in food intake. Second, previous research with the same design (Berdanier et al.) found no difference in food intake with a similar weight gain pattern as was found in our study. Although they used a different rat model of T2D, the reduction in weight gain in the diabetic rats consuming the egg-based diet, provided at the same level as we employed, was similar in magnitude. More importantly, and key to our conclusions, they found no significant difference in food intake patterns between diabetic groups fed whole egg-based compared to casein-based diets in a study that was conducted for a much longer period of time than the 8 weeks reported here.

Our laboratory recently demonstrated that excessive urinary excretion of 25D–DBP and albuminuria occurred in rats with type 1 diabetes and T2D. Feeding diabetic rats high-amylose maize partially resistant to digestion prevented excretion of albumin and 25D–DBP, suggesting that vitamin D balance in T2D can be maintained by dietary resistant starch by its nephroprotective actions without vitamin D supplementation (6, 23). Because we did not expect whole egg to be nephroprotective, it is not surprising that whole egg did not impact urinary loss of 25D. In the future, investigating the impact of a diet comprised of both resistant

starch and whole egg may yield more robust outcome with respect to vitamin D balance and glucose homeostasis, than either dietary component individually.

In summary, this study demonstrates that a whole egg-containing diet is a highly effective strategy to maintain circulating 25D concentrations in T2D. Future work will include a dose-response study to determine the minimal amount of whole egg required to provide beneficial effects with respect to maintaining vitamin D balance. Because the ZDF rat represents an extreme model of T2D relative to humans, it can be theorized that the benefits of whole egg on vitamin D balance could be produced clinically by a diet containing much less whole egg than was used for this study. Additionally, direct measurements of body composition will be needed to better characterize the weight differences observed in diabetic rats fed whole egg compared to the other groups.

Authors' Contributions

S.K.J. performed all aspects of animal maintenance, preparation of experimental diets, and laboratory experiments as well as drafted the original version of this manuscript. G.Y.K. assisted in animal maintenance and laboratory procedures. K.L.S. and M.J.R. assisted with the study design. All authors read and approved the final manuscript.

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Tables and Figures

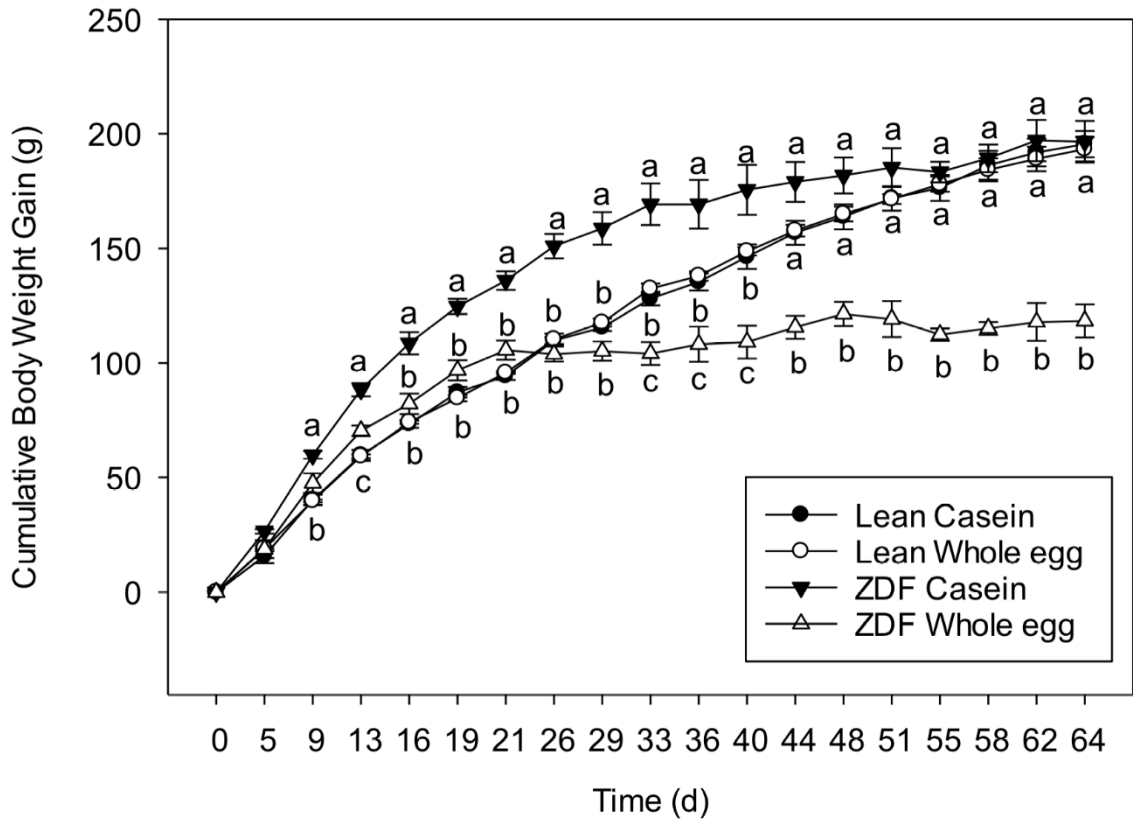


Figure 1. Whole egg consumption reduced cumulative weight gain in Zucker diabetic fatty (ZDF) rats. ZDF rats and their lean controls were fed either a casein- or whole egg-based diet for 8 weeks. Weights were recorded daily and are reported as cumulative weight gain from d 0. Data are means \pm SEM (n=5). At each time point, values without a common letter are statistically significant ($P < 0.05$).

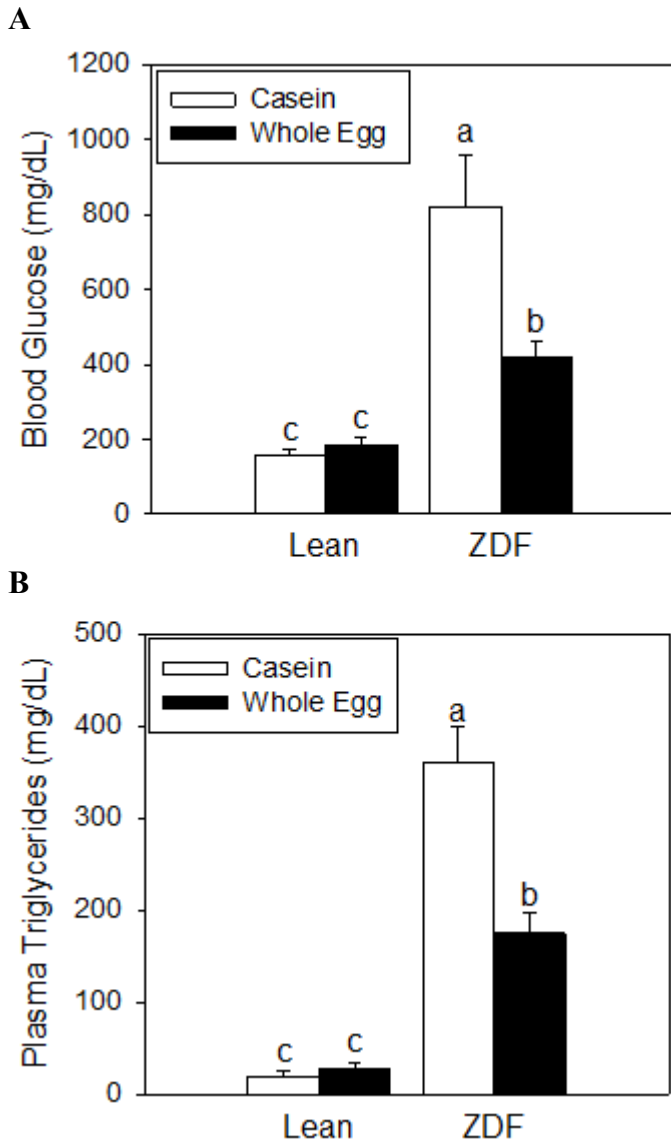


Figure 2. Whole egg consumption reduced characteristic elevations in blood glucose (A) and triglyceride concentrations (B) in Zucker diabetic fatty (ZDF) rats. Male ZDF rats and their lean controls were fed either a casein- or whole-egg based diet for 8 weeks. At the end of the treatment period rats were anesthetized, and whole blood was collected by cardiac puncture. Blood glucose concentrations were measured at the time of sacrifice using a glucometer. Triglyceride concentrations were analyzed using a commercial colorimetric kit. Data are means \pm SEM (n=5). Bars without a common letter are statistically significant ($P < 0.05$).

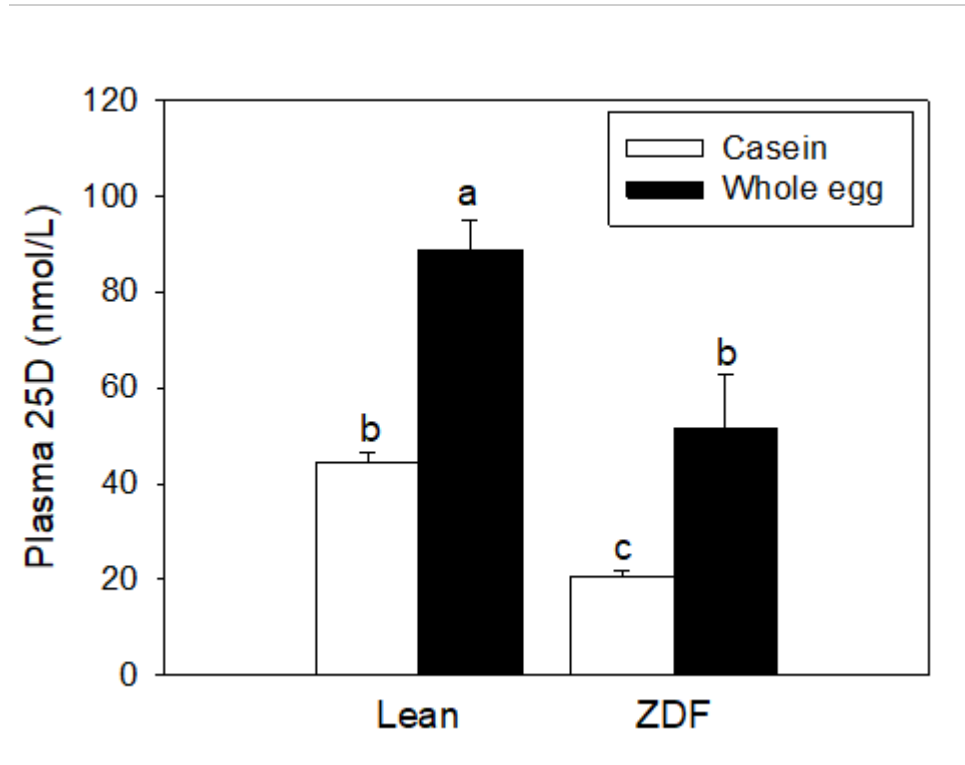


Figure 3. Whole egg consumption increased circulating 25-hydroxyvitamin D concentrations in Zucker diabetic fatty (ZDF) and lean control rats. Male ZDF rats and their lean controls were fed either a casein- or whole-egg based diet for 8 weeks. At the end of the treatment period rats were anesthetized, and whole blood was collected by cardiac puncture. 25D concentrations were measured using a commercial enzyme immunoassay kit. Data are means \pm SEM (n=5). Bars without a common letter are statistically significant ($P < 0.05$).

Table 1. Composition of casein-based and whole egg-based diets fed to lean control and Zucker Diabetic Fatty rats for 8 weeks

ingredient ¹	casein-based	whole egg-based
	g/kg	
casein (vitamin free)	200	0
dried standard whole egg type 350 ^{2,3}	0	408
cornstarch	437	392
glucose (monohydrate)	150	150
mineral mix (AIN 93)	35	35
vitamin mix (AIN 93) ³	10	10
corn oil	163	0
choline bitartrate	2	2
L-methionine	3	3

¹ Ingredients were purchased from Harlan Teklad (Madison, WI) with the exception of dried whole egg (Rose Acre Farms, Guthrie Center, IA) as well as L-methionine and choline bitartrate (Sigma Aldrich, Milwaukee, WI).

² Total protein and lipid content provided by 408 g of whole egg is 49% (200 g) and 40% (163 g), respectively.

³ Total vitamin D provided by casein-based and whole egg-based diets are 25 and 37.6 µg/kg diet, respectively.

Table 2. Final body weights and relative tissue weights of lean control and diabetic Zucker Diabetic Fatty (ZDF) rats fed a casein or whole egg-based diet for 8 weeks ¹

	lean		ZDF	
	casein	whole egg	casein	whole egg
final body weight (g)	358 ± 11 ^{a,b}	377 ± 11 ^a	400 ± 15 ^a	320 ± 12 ^b
liver (g/100 g body weight)	3.01 ± 0.35 ^b	3.30 ± 0.15 ^b	5.52 ± 0.19 ^a	6.00 ± 0.06 ^a
kidney (g/100 g body weight)	0.65 ± 0.04 ^b	0.56 ± 0.02 ^b	0.80 ± 0.04 ^a	0.84 ± 0.04 ^a
adipose (g/100 g body weight)	0.47 ± 0.10 ^b	0.58 ± 0.11 ^b	2.03 ± 0.40 ^a	1.83 ± 0.15 ^a

¹ Values are the mean ± SEM (*n* = 5).

CHAPTER 4. DIETARY WHOLE EGG CONSUMPTION ATTENUATES BODY WEIGHT GAIN AND IS MORE EFFECTIVE THAN SUPPLEMENTAL CHOLECALCIFEROL IN MAINTAINING VITAMIN D BALANCE IN TYPE 2 DIABETES

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Abstract

Background: Type 2 diabetes (T2D) is characterized by vitamin D insufficiency owing to excessive urinary loss of 25-hydroxycholecalciferol (25(OH)D). We previously reported that a diet containing dried whole egg, a rich source of vitamin D, was effective at maintaining circulating 25(OH)D concentrations in T2D rats. Furthermore, whole egg consumption reduced body weight gain in T2D rats.

Objective: This study was conducted to compare whole egg consumption to supplemental cholecalciferol with respect to vitamin D balance, weight gain, and body composition in T2D rats.

Methods: Male Zucker diabetic fatty (ZDF) rats (n= 24) and their lean controls (n=24) were obtained at 5 wk of age and randomly assigned to 3 treatment groups: a casein-based diet (CAS), a dried whole egg-based diet (WE), or a casein-based diet containing supplemental cholecalciferol (CAS+D) at the same level of cholecalciferol provided by the dried whole egg-based diet (37.6 µg/kg diet). Rats were fed their respective diets for 8 wk. Weight gain

and food intake were measured daily, circulating 25(OH)D concentrations were measured by ELISA, and body composition was analyzed by dual X-ray absorptiometry.

Results: Weight gain and percent body fat were reduced by approximately 20% and 11%, respectively, in ZDF rats fed WE compared to ZDF rats fed CAS or CAS+D. ZDF rats fed CAS had 21% lower serum 25(OH)D concentrations than lean rats fed CAS. In ZDF rats, WE consumption increased serum 25(OH)D concentrations 130% compared to CAS, whereas consumption of CAS+D increased serum 25(OH)D concentrations 35% compared to CAS.

Conclusion: Our data suggest that dietary consumption of whole egg is more effective than supplemental cholecalciferol in maintaining circulating 25(OH)D concentrations in T2D rats. Moreover, whole egg consumption attenuated weight gain and reduced percent body fat in ZDF rats. These data may support new dietary recommendations targeting prevention of vitamin D insufficiency in T2D.

Introduction

Although vitamin D insufficiency is common globally, it is highly predominate in type 2 diabetes (T2D), affecting 70-90% of the T2D population (1-3). Vitamin D insufficiency is defined as circulating 25-hydroxycholecalciferol (25(OH)D) concentrations between 30-50 nmol/L (12-20 ng/mL), whereas deficiency is defined as serum 25(OH)D concentrations below 30 nmol/L (12 ng/mL). Evidence from prospective studies suggests a correlation between inadequate vitamin D concentrations and T2D (4-6). Specifically, vitamin D deficiency may be a factor in the development of insulin resistance as well as the pathogenesis of T2D by affecting either insulin sensitivity, β -cell function or both (7-9); however, other studies have found little or no association between T2D and these measures

(10, 11). Furthermore, we have found that diabetic nephropathy, a well-characterized complication of T2D, leads to excessive urinary excretion of circulating 25(OH)D and vitamin D binding protein (DBP), thereby exacerbating vitamin D deficiency (12-14). Therefore, there is a critical need to identify dietary intervention strategies to prevent and/or treat vitamin D deficiency in the diabetic population.

Dietary vitamin D exists in two forms, ergocalciferol (vitamin D2) and cholecalciferol (vitamin D3). The most abundant dietary form is cholecalciferol, which is metabolized in the liver to 25(OH)D by humans and animals; therefore, animal-based foods are a source of 25(OH)D as well as cholecalciferol. The precursor form of active vitamin D is 25(OH)D, which, in the circulation, reflects an individual's vitamin D status. Vitamin D recommendations for diabetics are inconsistent. Randomized clinical trials focusing on T2D outcomes vary with respect to vitamin D dose and regimen, ranging from 1000-6000 IU/ d to 20,000-40,000 IU /wk with study durations lasting from several months to years. Results from these studies differ with respect to improvements in fasting blood glucose, glycated hemoglobin (HbA1c), and insulin sensitivity (15-19). While the current RDA for vitamin D in adults is 600 IU/ d, supplementation guidelines remain an intense topic of debate. Although an intake of 600 IU/d is sufficient to support musculoskeletal health, more studies are needed to clearly assess the impact of supplementary cholecalciferol on chronic diseases (20). As reviewed by Mathieu (21), growing evidence supports the adoption of the international guidelines on supplementation of cholecalciferol at 500-1000 IU/d to prevent vitamin D deficiency and reduce the risk of T2D onset.

Treatment of T2D is primarily focused on lifestyle modifications, including improvements in diet and physical activity, to promote weight loss and improve blood

glucose control. We have previously shown that dietary resistant starch was an effective dietary strategy for maintaining vitamin D balance by protecting renal health, thereby preventing urinary excretion of 25(OH)D and DBP. In contrast, the present study utilized dried whole eggs to focus on increasing dietary consumption of vitamin D as a means to improve vitamin D status. Whole eggs are an excellent source of vitamin D, in the form of both 25(OH)D and cholecalciferol, which is found exclusively in the yolk (22). Promoting egg consumption has been a controversial diet recommendation for individuals with T2D because of the rich cholesterol content of eggs. Because diabetics are at an increased risk for cardiovascular disease (CVD), they have been encouraged to limit the number of eggs they consume. To date, there are a number of studies that contradict the relation between egg consumption and chronic disease (23-27). More importantly, recent revisions to the Dietary Guidelines for Americans no longer include recommendations to limit intake of dietary cholesterol, a decision based on the growing body of research showing that dietary cholesterol intake has little effect on serum cholesterol concentrations and subsequent health risks (28). Furthermore, numerous human studies report that egg consumption is associated with increased satiety, which leads to reduced overall caloric intake (29-33). Some human studies also report that egg consumption promotes weight loss; however, the literature regarding the effect of egg consumption on body weight management remains inconsistent (34, 35). Nevertheless, a growing body of research demonstrates several benefits of whole egg consumption, such as the high nutrient content and satiating effect of whole eggs; thus, dietary whole egg consumption may be beneficial in the diabetic population (36-38).

We previously reported that a dried whole egg-containing diet is a highly effective strategy to maintain circulating 25(OH)D concentrations in T2D rats (39). Additionally,

whole egg consumption reduced weight gain in diabetic rats. Thus, the primary objectives of this follow-up study were 1) to compare the vitamin D provided by whole eggs to a diet supplemented with cholecalciferol in maintaining serum vitamin D balance and 2) to further investigate the effect of whole egg consumption on weight gain and body composition in T2D rats.

Materials and Methods

Animals and Diets. All animal studies were approved by the Institutional Animal Care and Use Committee at Iowa State University and were performed according to the Iowa State University Laboratory Animal Resources Guidelines. Male Zucker diabetic fatty (ZDF; fa/fa) rats (n= 24) and lean (fa/+) control rats (n= 24) were purchased at 5 wk of age (Charles River Laboratories). Rats were housed individually in plastic cages in a temperature-controlled room with a 12-h light-dark cycle. All diets were formulated and pelleted by Research Diets, Inc. Dried whole egg was purchased from Rose Acre Farms and sent to Research Diets, Inc. for diet formulation. All rats were acclimated to a semi-purified diet (AIN-93G) for 1 wk. Rats were randomly assigned to 1 of 3 experimental diets (Figure 1): a casein-based diet (CAS), a dried whole egg-based diet (WE), or a casein-based diet containing supplemental cholecalciferol (CAS+D) provided at the same level of cholecalciferol supplied by the WE diet (37.6 µg/kg diet). Vitamin mix in all diets provided 25 µg vitamin D/kg diet. The whole egg diet contained an additional 12.6 µg cholecalciferol/kg diet, thus, the WE diet provided a total of 37.6 µg cholecalciferol/kg diet. This level was matched in the CAS+D diet with the addition of 12.6 µg cholecalciferol. All diets provided protein at 20% (w/w) and were matched for lipid content (18.3%) via the addition of corn oil to the CAS and CAS+D diets, accounting for the additional lipid provided by the dried whole egg. Rats were given ad

libitum access to food and water for 8 wk. Food intake was recorded daily for each rat beginning at wk 3 of the study. Pelleted diets were weighed and distributed daily. Consumption was defined as the difference in pellet weight within a 24 h period. Prior to sacrifice, rats were placed in metabolic cages for 12 h, during which urine was collected and then stored at -80°C for subsequent analysis. Rats were anesthetized via a single intraperitoneal injection of ketamine:xylazine (90:10 mg/kg body weight). Whole blood was collected via cardiac puncture and blood glucose was measured using a glucometer (Bayer Healthcare). Body fat, lean body mass, bone mineral density and bone mineral content were measured post-necropsy using dual energy x-ray absorptiometry (DEXA).

Biochemical Analysis. Analysis of serum and urinary creatinine was measured using commercially available colorimetric kits (Cayman Chemical). Urinary total protein concentrations were measured using a bicinchoninic acid assay (Thermo Scientific Pierce), serum concentrations of 25(OH)D were analyzed using a commercial enzyme immunoassay kit (Immunodiagnostic Systems), and urinary concentrations of 25(OH)D and DBP were also analyzed using a commercial enzyme immunoassay kit (Immunodiagnostic Systems and Life Diagnostics, respectively) as previously described (12, 13, 40). Authenticity of all kits for use on rodent biological samples has been verified by the manufacturer.

Statistical Analysis. All data were analyzed using SigmaPlot 9.0 (Systat Software Inc.). Mean values were evaluated for statistically significant differences ($P < 0.05$) using a two-way ANOVA (genotype x diet) followed by the Fisher's Least Significant Difference (LSD)

post hoc test for multiple comparisons. Nonparametric analysis was utilized when normality failed or variances were unequal using a Kruskal-Wallis one-way ANOVA on Ranks.

Results

Whole egg consumption reduced total body weight and cumulative weight gain despite increased food intake in ZDF rats. Lean and ZDF rats initially gained the same amount of weight across all dietary groups within a given genotype. As expected, ZDF rats fed the CAS and CAS+D diets gained more weight throughout the study compared to all lean control rats (Figure 1A). However, ZDF rats fed WE exhibited a plateau in cumulative weight gain beginning on d 10 and an approximate 20% reduction in weight gain compared to ZDF rats fed CAS and CAS+D after 8 wk of dietary treatment. Furthermore, cumulative weight gain in ZDF rats fed WE was statistically equivalent to all lean control rats beginning on d 22, and for the remainder of the study. Although ZDF rats fed WE gained less weight than ZDF rats fed CAS and CAS+D, total food intake in ZDF rats fed WE was approximately 7% higher compared to ZDF rats fed CAS and CAS+D (Figure 2). Beginning at wk 5, ZDF rats fed WE had higher weekly food intake per 100 g body weight compared to ZDF rats fed CAS and CAS+D, whereas weekly food intake (g/100 g body weight) did not differ in lean rats regardless of diet (Figure 1B). Moreover, cumulative weight gain and total food intake did not differ between the lean control rats fed either CAS, CAS+D or WE. There were no differences in the food efficiency ratio within the ZDF genotype. In contrast, the food efficiency ratio [(weight gain, g/food intake, g) x 100.] was 12% lower in lean rats fed WE compared to lean rats fed CAS (Table 2).

ZDF rats fed WE exhibited a lower body fat percentage than ZDF rats fed CAS and CAS+D. Percent body fat and percent lean body mass are presented in Figure 2. Percent body fat did not differ between dietary groups within lean control rats. In contrast, WE consumption in ZDF rats reduced body fat percentage by 8 and 13%, respectively, compared to ZDF rats fed CAS and CAS+D. Lean body mass was increased by 11% in ZDF rats fed WE compared to ZDF rats fed CAS+D, whereas lean body mass did not differ, regardless of diet, within the lean rats. Bone mineral density did not differ across all dietary groups and genotypes (Table 2). Bone mineral content, expressed as a percentage of body weight, did not differ between dietary groups within the lean control rats. Because bone mineral content was corrected for body weight, bone mineral content was 7 and 9% higher, respectively, in WE-fed ZDF rats than in ZDF rats consuming CAS or CAS+D.

The WE diet elevated circulating 25(OH)D concentrations to a greater extent than the CAS+D diet. Serum 25(OH)D concentrations of all treatment groups are shown in Figure 3. As expected, ZDF rats fed CAS had lower (21%) serum 25(OH)D concentrations than their lean counterparts fed CAS. The WE diet increased 25(OH)D concentrations by 130% compared to ZDF rats fed CAS, whereas CAS+D increased circulating 25(OH)D by only 35% compared to ZDF rats fed CAS. Likewise, serum 25(OH)D concentrations of lean rats fed CAS+D and the WE diet were increased by 19% and 113%, respectively, compared to lean rats fed CAS. When compared to CAS+D, WE increased serum 25(OH)D concentrations by 80% and 70% in lean and ZDF rats, respectively.

Serum and urinary biochemical measurements. The presence of hyperglycemia in ZDF rats confirmed the diabetic state; however, blood glucose did not differ between dietary groups within the lean or ZDF genotype. In lean rats fed WE, serum insulin was lower than all other dietary groups. The homeostatic model assessment of insulin resistance (HOMA-IR) was decreased by 80 and 71%, respectively, in lean rats fed WE compared to lean rats fed CAS and CAS+D. HOMA-IR values did not differ between dietary groups within the ZDF genotype. Urinary output, urinary 25(OH)D, urinary DBP and serum creatinine were increased in ZDF rats compared to lean rats. Urinary creatinine excretion was reduced in ZDF rats fed CAS and CAS+D by approximately 67% and 79%, respectively, compared to all lean rats. In contrast, urinary creatinine excretion did not differ in ZDF rats fed the WE diet compared to all lean rats. Urinary total protein excretion did not differ between lean and ZDF rats. WE consumption was without effect on urinary measures within the lean or ZDF genotype. Likewise, there were no differences in serum creatinine within lean or ZDF rats Table 3.

Discussion

We have previously shown that a dried whole egg-based diet is a highly effective strategy for maintaining serum 25(OH)D concentrations in rats with T2D (39). The present study demonstrates that vitamin D derived from whole egg may be more effective than an equivalent amount of supplemental cholecalciferol added to a casein-based diet at maintaining serum 25(OH)D concentrations. Serum 25(OH)D concentrations were markedly higher in both lean and ZDF rats fed WE compared to rats fed CAS+D. Consumption of the WE diet in ZDF rats resulted in elevated serum 25(OH)D despite urinary losses due to the presence of diabetic nephropathy. All ZDF rats exhibited excessive urinary excretion of

25(OH)D regardless of dietary group, which suggests that the increase in serum 25(OH)D in ZDF rats fed the WE diet was due to a mechanism other than attenuated urinary losses. The difference in serum 25(OH)D concentrations between the WE and CAS+D diets may be due to the potency of 25(OH)D contained within whole eggs. In support of this theory, Cashman et al. carried out a human study comparing orally supplemented 25(OH)D to cholecalciferol and found that oral supplementation with 25(OH)D raised serum 25(OH)D concentrations five times more than an oral cholecalciferol supplement per microgram consumed (41). As reviewed by Ovesen et al., a number of studies have reported 25(OH)D to be more potent than the equivalent amount of cholecalciferol in raising serum concentrations of 25(OH)D, however, the exact potency factor remains undetermined (42).

Nutritionally, eggs boast a number of benefits; they are rich in high quality protein, contributing to satiety; contain a high nutrient-to-energy density ratio, and are inexpensive and easy to prepare (24, 43). Furthermore, egg consumption has been shown to increase circulating HDL-cholesterol concentrations, which is associated with lower CVD risk (44-46). Despite these advantages, there remains a negative perception toward egg consumption for individuals with diabetes. Previous studies have suggested that high egg consumption may be associated with higher CVD outcomes in people with T2D, a population already at risk for CVD (47-49); however, more recent studies contradict this finding. A randomized control trial found that consuming 2 eggs per d for 3 mo did not negatively affect the lipid profile of diabetics (23). A similar study reported that egg consumption, in combination with healthy dietary changes, improved glucose homeostasis, as well as lipid profiles in a diabetic population (45). Furthermore, the 2015 Dietary Guidelines for Americans no longer include recommendations to limit intake of dietary cholesterol as a direct result of the decades of

research demonstrating little effect of dietary cholesterol on serum cholesterol concentrations and subsequent health risks (50). Taken together, egg consumption, as a source of vitamin D, represents a reliable dietary intervention strategy for maintaining serum 25(OH)D concentrations in diabetics without posing additional heart health risks.

We previously reported that whole egg consumption attenuated weight gain in ZDF rats fed a dried whole egg-based diet compared to ZDF rats fed a casein-based diet (39). In the present study, ZDF rats fed WE exhibited a marked reduction in cumulative body weight gain compared to ZDF rats fed CAS and CAS+D. Furthermore, cumulative body weight gain in ZDF rats fed WE was the same as all lean control rats; thus, the reduction in cumulative body weight gain by WE is genotype specific, only occurring in the obese, diabetic state. The observed decrease in body weight in ZDF rats fed WE was, in part, due to a decrease in body fat percentage compared to ZDF rats fed CAS and CAS+D. In our recent unpublished observations using a diet-induced model of obesity in Sprague Dawley rats, cumulative weight gain was decreased by 23% in diet-induced obese rats fed a dried whole egg-based diet compared to diet-induced obese rats fed a casein-based diet. Moreover, diet-induced obese rats fed the dried whole egg-based diet gained the same amount of weight as control rats fed casein- and dried whole egg-based diets. These findings support the concept that whole egg consumption reduces weight gain in an obese state in both genetic and diet-induced models, whereas whole egg consumption is without effect on body weight in a lean phenotype. Previous studies have attributed differences in body weight following a whole egg-based diet to increased satiety, while others have found no difference in food intake (29-33). However, in the present study, we report an increase in food intake in ZDF rats fed WE, suggesting that the reduction in body weight in the ZDF genotype is likely the result of a

mechanism other than satiety. Furthermore, we found no difference in the food efficiency ratio in ZDF rats within any of the dietary groups. Others have suggested dietary fat as a potential mechanism and there is evidence to support that dietary fat composition may influence final body weight or weight gain in an obese state, depending on the ratio of unsaturated to saturated fatty acids (51, 52). Other potential mechanisms include changes in thermogenesis or energy expenditure and alterations in the gut microbiome. Several rodent and human studies have found an association between obesity and modifications to the intestinal microbiota; thus, it is possible that a component of the WE diet interacts with the intestinal microbiota in an obese state only (53, 54). Further studies are needed to elucidate the mechanism by which whole egg consumption attenuates weight gain in both the genotype- and diet-induced obese phenotype.

In conclusion, the present study demonstrates that dietary consumption of whole egg may be more effective than supplemental cholecalciferol in maintaining normal circulating 25(OH)D concentrations in T2D. Furthermore, whole egg consumption results in reduced body weight gain in obese, type 2 diabetic rats. Future dose response studies are required to identify the minimal amount of dietary whole egg required to maintain vitamin D homeostasis and attenuate body weight gain in obesity and T2D. Our findings support the concept that inclusion of whole eggs in the diet is an important recommendation for maintenance of vitamin D balance in T2D.

Acknowledgments

C.J.S and S.K.J. performed all aspects of animal maintenance, administration of experimental diets, laboratory experiments and drafted the original version of this manuscript. K.E.H. and C.H.R assisted in animal maintenance and laboratory procedures. K.L.S. and M.J.R. assisted

with the study design and edits to the manuscript. All authors read and approved the final version of this manuscript.

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Tables and Figures

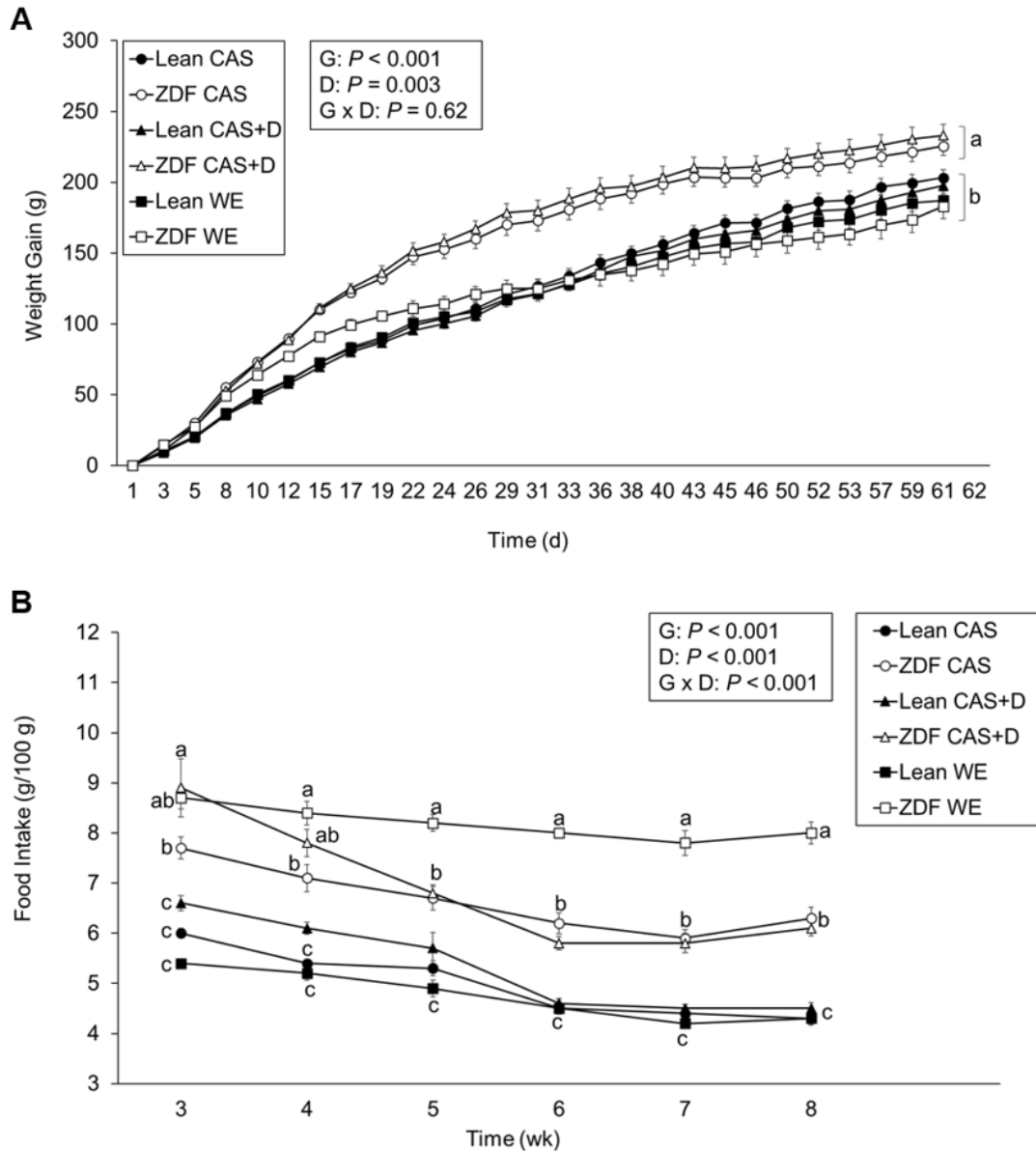


Figure 1. Cumulative body weight gain (A) and food intake (B) in lean control and Zucker diabetic fatty (ZDF) rats fed a casein-based (CAS), whole egg-based (WE), or casein-based diet including supplemental cholecalciferol (CAS+D) for 8 wk. Data are mean values \pm SEMs; $n = 8$. Values without a common letter differ ($P < 0.05$). (B) For clarity, P values reported are for wk 8. G; genotype, D; diet, G x D; genotype x diet (interaction).

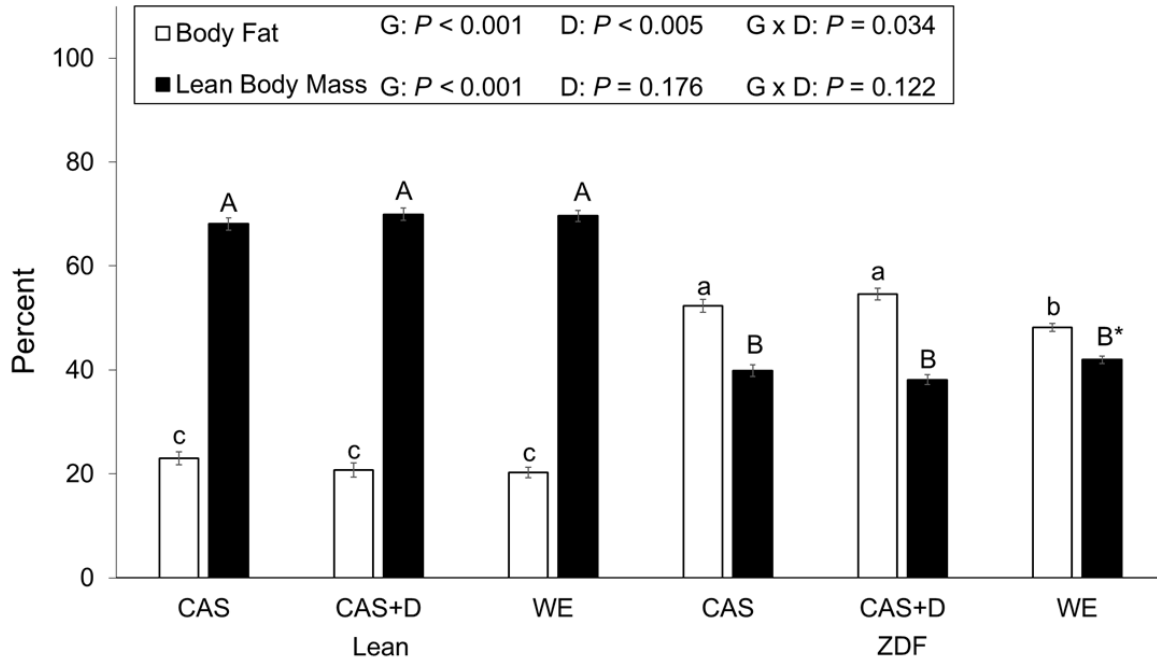


Figure 2. Percent body fat and lean body mass of lean controls and Zucker diabetic fatty (ZDF) rats following 8 wk dietary treatment with a casein-based (CAS), whole egg-based (WE), or casein-based diet including supplemental cholecalciferol (CAS+D). Data are means \pm SEMs; $n = 8$. Bars without a common letter differ ($P < 0.05$). Capital letters indicate differences in lean body mass and lower case letters indicate differences in percent body fat. An asterisk denotes a difference in lean body mass between ZDF rats fed WE and ZDF rats fed CAS+D when analyzed by a one-way ANOVA within the ZDF genotype. G; genotype, D; diet, G x D; genotype x diet (interaction).

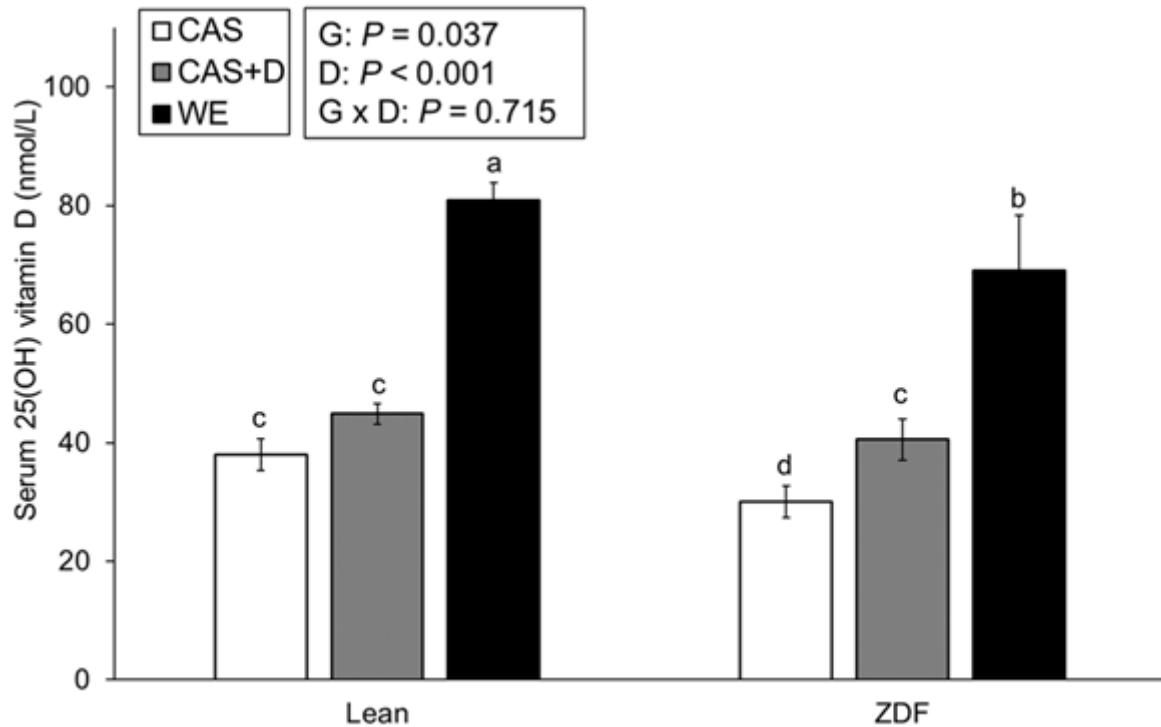


Figure 3. Circulating 25-hydroxycholecalciferol (25(OH)D) concentrations of lean control and Zucker diabetic fatty (ZDF) rats following 8 wk dietary treatment with a casein-based (CAS), whole egg-based (WE), or casein-based diet including supplemental cholecalciferol (CAS+D). Data are means \pm SEMs; $n = 8$. Bars without a common letter differ ($P < 0.05$). G; genotype, D; diet, G x D; genotype x diet (interaction).

Table 1. Composition of the casein-based diet (CAS), casein-based diet including supplemental cholecalciferol (CAS+D) and whole egg-based diet (WE) fed to lean control and Zucker diabetic fatty rats for 8 wk¹

Ingredient	CAS	CAS+D <i>g/kg</i>	WE
Casein (vitamin-free)	200	200	0
Dried standard whole egg, Type 350 ^{2,3,4}	0	0	435
Cornstarch	417	417	365
Glucose monohydrate	150	150	150
Mineral Mix (AIN 93)	35	35	35
Vitamin Mix (AIN 93)	10	10	10
Cholecalciferol, 100,000 IU/g	0	0.00504	0
Biotin, 1%	0	0	0.4
Corn oil	183	183	0
Choline bitartrate	2	2	2
L-Methionine	3	3	3

¹All diets were formulated by and purchased from Research Diets Inc.

² Whole egg was purchased from Rose Acre Farms and sent to Research Diets Inc. for diet formulation.

³ Total protein and lipid content provided by 435 g of whole egg were 46% (200 g) and 42% (183 g), respectively.

⁴ Total cholecalciferol provided by the casein-based diet, casein-based diet including supplemental cholecalciferol and whole egg-based diet were 25, 37.6 and 37.6 $\mu\text{g}/\text{kg}$ diet, respectively.

¹ Data are means \pm SEMs; n=8. Mean values within a row without a common letter are statistically significant ($P < 0.05$).

Table 2. Final body weight, total food intake, bone mineral density, bone mineral content, and food efficiency ratio of lean control and Zucker diabetic fatty rats (ZDF) fed a casein-based diet (CAS), a casein-based diet including supplemental cholecalciferol (CAS+D) and a whole egg-based diet (WE) for 8 wk¹

Parameter	Lean			ZDF			<i>P</i>		
	CAS	CAS+D	WE	CAS	CAS+D	WE	Genotype	Diet	Genotype x Diet
Final body weight, g	351 ± 5 ^c	338 ± 11 ^c	322 ± 19 ^c	425 ± 5 ^a	419 ± 5 ^a	383 ± 5 ^b	<0.001	0.003	0.62
Total food intake, g	419 ± 6 ^c	430 ± 13 ^c	400 ± 9 ^c	748 ± 14 ^b	751 ± 21 ^b	803 ± 12 ^a	<0.001	0.419	0.006
Bone mineral density, g/cm ²	0.20 ± 0.003	0.19 ± 0.004	0.19 ± 0.005	0.19 ± 0.003	0.18 ± 0.003	0.19 ± 0.007	0.188	0.057	0.188
Bone mineral content, (% of body weight)	2.59 ± 0.03 ^a	2.65 ± 0.03 ^a	2.68 ± 0.03 ^a	2.15 ± 0.05 ^c	2.11 ± 0.03 ^c	2.29 ± 0.02 ^b	<0.001	0.002	0.089
Food efficiency ratio	25 ± 0.5 ^a	24 ± 0.9 ^a	22 ± 0.7 ^b	10 ± 0.4 ^c	11 ± 0.9 ^c	9 ± 0.6 ^c	0.037	<0.001	0.715

¹ Data are means ± SEMs; n=8. Mean values within a row without a common letter are statistically significant (*P* < 0.05).

Table 3. Biochemical measurements of lean control and Zucker diabetic fatty (ZDF) rats fed a casein-based diet (CAS), a casein-based diet including supplemental cholecalciferol (CAS+D) and a whole egg-based diet (WE) for 8 wk ¹

Biochemical Measurement	Lean			ZDF			<i>P</i>		
	CAS	CAS+D	WE	CAS	CAS+D	WE	Genotype	Diet	Genotype x Diet
Urinary output, mL	8.7 ± 3.6 ^b	6.8 ± 1.5 ^b	3.1 ± 1.0 ^b	14.6 ± 1.5 ^a	12.9 ± 1.8 ^a	19.9 ± 4.0 ^a	<0.001	0.71	0.043
Urinary total protein, mg/ 12	29 ± 3	48 ± 13	53 ± 5	38 ± 4	32 ± 6	37 ± 7	0.189	0.29	0.12
Urinary creatinine, mg/12 h	3.3 ± 0.3 ^a	3.8 ± 0.5 ^a	2.8 ± 0.5 ^a	1.1 ± 0.2 ^b	0.7 ± 0.1 ^b	1.6 ± 0.4 ^{ab}	<0.001	0.991	0.029
Serum creatinine, mg/dL	2 ± 0.3 ^b	2 ± 0.3 ^b	2 ± 0.2 ^b	17 ± 4 ^a	21 ± 7 ^a	21 ± 6 ^a	<0.001	0.968	0.929
Urinary 25(OH)D, pmol/mg creatinine	66 ± 15.9 ^b	64 ± 21.1 ^b	46 ± 7.6 ^b	1610 ± 691 ^a	1700 ± 654 ^a	1990 ± 1070 ^a	<0.001	0.95	0.938
Urinary DBP, µg/12 h	0.910 ± 0.19 ^b	0.983 ± 0.28 ^b	1.01 ± 0.17 ^b	993 ± 213 ^a	749 ± 253 ^a	1270 ± 443 ^a	<0.001	0.523	0.523
Blood glucose, mg/dL	256 ± 21 ^b	291 ± 17 ^b	284 ± 24 ^b	688 ± 41 ^a	560 ± 67 ^a	693 ± 43 ^a	<0.001	0.261	0.093
Serum insulin, ng/mL	3.8 ± 0.7 ^a	2.3 ± 0.2 ^a	0.8 ± 0.4 ^b	2.9 ± 0.4 ^a	3.7 ± 0.4 ^a	2.5 ± 0.7 ^a	0.082	0.004	0.018
HOMA-IR, %	54 ± 9 ^{ab}	38 ± 4 ^b	11 ± 13 ^c	113 ± 21 ^a	117 ± 21 ^a	99 ± 29 ^a	<0.001	0.161	0.647

¹ Data are means ± SEMs; *n* = 8. Mean values within a row without a common letter are statistically significant (*P* < 0.05).

CHAPTER 5. WHOLE EGG CONSUMPTION EXERTS A NEPHROPROTECTIVE EFFECT IN AN ACUTE RODENT MODEL OF TYPE 1 DIABETES

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*CJS and SKJ contributed equally to this work.

Abstract

Nephropathy is a well characterized complication of type 1 diabetes (T1D), resulting in proteinuria and urinary loss of micronutrients. We previously found that a whole egg-based diet maintained vitamin D balance in type 2 diabetic rats despite excessive urinary losses due to nephropathy. The goal of this study was to investigate the impact of whole egg consumption in T1D rats. Sprague-Dawley rats were randomly assigned to T1D or non-diabetic control groups and fed a casein or whole egg-based diet for 32 days. On day 26, two-thirds of the rats received a streptozotocin injection to induce T1D. Whole egg consumption attenuated polyuria, proteinuria and renal hypertrophy in T1D rats. These data suggest that dietary intervention with whole egg may offer renal protection in T1D.

Introduction

Type 1 diabetes (T1D) is an autoimmune-mediated disorder, which results in destruction of the pancreatic β -cells and a lack of endogenous insulin production. Diabetic nephropathy is a well-characterized microvascular complication of T1D, affecting approximately 32-44% of the type 1 diabetic population (1-3). Furthermore, diabetes

accounts for nearly half of reported cases of chronic kidney disease in the United States and worldwide (4). Key events in the pathophysiology of diabetic nephropathy include microalbuminuria and glomerular hyperfiltration, which may progress to overt proteinuria and a decreased glomerular filtration rate (GFR). While urinary protein excretion and impaired GFR are often concomitant in their presentation, they are separate manifestations of diabetic nephropathy and thus, do not always follow a sequential pattern (5). Renal structural changes such as basement membrane thickening, glomerular hypertrophy, and mesangial expansion are also hallmark characteristics of diabetic nephropathy (6)

Vitamin D deficiency, defined as serum 25-hydroxycholecalciferol (25D) concentrations less than 12 ng/mL (i.e., 30 nmol/L), is prevalent in the diabetic population (7-10). A major factor associated with low vitamin D status in the diabetic population is compromised renal function. In the circulation, 25D is bound to its transporter, vitamin D binding protein (DBP). The 25D-DBP complex is taken up by the kidney via megalin-mediated endocytosis into the renal proximal tubule where it may undergo hydroxylation to 1,25-dihydroxycholecalciferol (1,25D), the active form of vitamin D, or be returned to circulation. Renal reabsorption of the 25D-DBP complex is compromised in diabetic nephropathy, resulting in excessive urinary excretion of 25D and DBP, which leads to insufficient 25D concentrations in the circulation (11-14).

Dietary strategies to optimize nutritional status in the diabetic population are of high importance. We have focused on dietary intervention strategies to improve vitamin D status by 1) increasing dietary intake; or 2) reducing urinary loss of 25D. We have reported that dietary resistant starch exerts a nephroprotective effect in rodent models of both T1D and type 2 diabetes (T2D), thereby preventing excessive urinary 25D excretion and maintaining

vitamin D balance (12, 13). Whole eggs are a rich dietary source of vitamin D, which is concentrated within the egg yolk. In whole eggs vitamin D is found in the form of both cholecalciferol (i.e., vitamin D₃) and 25D. We recently reported that a whole egg-containing diet is effective at maintaining vitamin D status in T2D rats (14). Furthermore, we have shown that a diet containing whole eggs may be more effective than a diet containing an equivalent amount of supplemental cholecalciferol at maintaining circulating 25D concentrations in T2D rats (15). Thus, the goal of this study was to determine the effect of whole egg consumption with respect to vitamin D balance and diabetic complications in an acute rodent model of T1D. Contrary to our studies in a T2D rat model, we did not observe vitamin D deficiency in an acute model of T1D; however, we did observe a nephroprotective effect that was not evident in a T2D model.

Materials and Methods

Chemicals. All chemicals were of analytical grade and purchased from commercial suppliers. Authenticity of all kits for use on rodent biological samples has been verified by the manufacturer.

Animals and Diets. All animal studies were approved by the Institutional Animal Care and Use Committee at Iowa State University and were performed according to the Iowa State University Laboratory Animal Resources Guidelines. Male Sprague-Dawley rats (N=18) were obtained at 7 weeks of age (Envigo, Madison, WI, USA). After one week of acclimation, rats were randomly assigned to either a modified AIN-93 casein-based (n=12) or a whole egg-based diet (n=6) (Table 1). All diets were prepared weekly; food and water was provided ad libitum. Both diets provided protein at 20% (w/w) and contained the same total

lipid content (16.3%, w/w) by the addition of corn oil to the casein-based diet to match the lipid contribution by the addition of whole egg. The vitamin mix in both diets provided 25 µg cholecalciferol/kg diet. The whole egg-based diet contained an additional 12.6 µg cholecalciferol/kg diet; thus, the whole egg-based diet provided a total of 37.6 µg cholecalciferol/kg diet. On day 26, half of the rats (n=6) fed the casein-based diet and all rats fed the whole egg-based diet (n=6) received a single intraperitoneal injection of freshly prepared STZ (60 mg/kg body weight) (Sigma Aldrich, St. Louis, MO, USA) in citrate buffer (10 mM, pH 4.5) to induce T1D; control rats were vehicle-injected. All rats were sacrificed 7 days after STZ or vehicle injection. Prior to sacrifice, rats were placed in metabolic cages for 12 hours to ensure that they were in a fasted state and for the collection of urine, which was stored at -80°C for subsequent analysis. Rats were anesthetized via a single intraperitoneal injection of ketamine:xylazine (90:10 mg/kg body weight), whole blood was collected by cardiac puncture, and kidneys were removed and weighed.

Biochemical Assessment. Analysis of urinary creatinine was measured using a commercially available colorimetric kit (Cayman Chemical, Ann Arbor, MI, USA). Urinary total protein concentrations were measured using a bicinchoninic acid colorimetric assay (Thermo Fisher Scientific, Waltham, MA, USA). Urinary concentrations of 25D and DBP were analyzed using commercial ELISA kits (Immunodiagnostic Systems, Gaithersburg, MD, USA, and Life Diagnostics, West Chester, PA, USA respectively), all as previously described (13-15).

Statistical Analysis. All data were analyzed using JMP Version 10.02 (SAS Institute Inc., Cary, NC, USA) software. T1D groups were compared to the control using Dunnett's test

(one-tailed, $P < 0.05$). Comparisons between the T1D groups fed the casein- versus whole egg-based diets were conducted using a Student's t-test ($P < 0.05$).

Results

Body Weight and Serum Parameters. Cumulative weight gain over the 4-week experimental period is shown in (Figure 1). As expected, upon STZ injection, body weight gain decreased significantly in the T1D rats compared to non-diabetic rats. At the conclusion of the study, T1D rats fed the casein-based and whole egg-based diets gained 19% and 21% less weight than healthy, non-diabetic rats, respectively (Table 2). Therefore, whole egg consumption was without effect on STZ-induced weight loss. T1D rats fed the casein-based and whole egg-based diets had blood glucose concentrations that were 147% and 112% higher compared to control rats, respectively (Table 2). Serum 25D concentrations were approximately 69% higher in T1D rats fed the whole egg-based diets compared to rats fed a casein-based diet, regardless of diabetes status (Table 2).

Renal and Urinary Parameters. Urine output, urinary total protein, 25D and DBP concentrations are presented in Figure 2. As expected, 12-hour urine output was 140% higher in T1D rats fed the casein-based diet compared to non-diabetic rats fed the casein-based diet (Figure 2A). However, whole egg consumption in T1D rats resulted in the same urine output as non-diabetic controls. Furthermore, urine output in T1D fed the whole egg-based diet was 54% lower compared to T1D rats fed the casein-based diet ($P = 0.0032$). Urinary total protein excretion was 412% and 156% higher, respectively, in T1D rats fed the casein- and whole egg-based diet compared to non-diabetic casein-fed rats. Notably, within T1D rats, whole egg consumption attenuated urinary total protein loss by 50% ($P = 0.0094$) compared

to the casein-based diet (Figure 2B). Urinary 25D concentrations were 294% higher in T1D rats fed the casein-based diet compared to non-diabetic casein-fed rats (Figure 2C). However, urinary 25D concentrations in T1D rats fed a whole egg-based diet did not differ from non-diabetic rats fed the casein-based diet. Similarly, urinary DBP concentrations were 1616% higher in T1D rats fed the casein-based diet compared to non-diabetic rats while urinary DBP excretion in T1D rats fed a whole egg-based diet did not differ from non-diabetic rats (Figure 2D). Relative kidney weight was increased in T1D rats fed the casein and whole egg-based diets by 59% and 32%, respectively, compared to non-diabetic rats, indicating renal hypertrophy. In T1D rats, the whole egg-based diet mitigated the increase in relative kidney weight by 14% ($P < 0.0021$) compared to the casein-based diet (Table 3). No significant differences in urinary creatinine excretion were observed between any of the treatment groups (Table 3).

Discussion

The present study demonstrated that inclusion of dried whole egg in the diet of T1D rats exerted a nephroprotective effect, as evidenced by decreased renal hypertrophy, and reduced polyuria and proteinuria. A similar pattern was observed with urinary excretion of 25D and DBP in T1D rats fed a whole egg-based diet, wherein excretion did not differ from either non-diabetic or T1D rats fed the casein-based diet. In clinical trials of chronic kidney disease, reduction of proteinuria is associated with a reduced rate of decline of kidney function (16-18). The risk of all-cause mortality increases with an increased degree of urinary albumin excretion (19, 20). Furthermore, increasing urinary albumin excretion is associated with a decline in GFR, although proteinuria and impaired GFR do not always present concomitantly (5). Because of the kidney's role in the filtration of metabolic end products

and nutrient reabsorption, the nutritional management of individuals with renal insufficiency is critical for reducing mortality and morbidity (21).

According to a review co-authored by the American Heart Association and the American Diabetes Association, cardiovascular events occur more frequently and earlier in patients with T1D than in nondiabetic populations (5). Although the precise mechanisms by which diabetes increases the likelihood of developing CVD are not completely defined, various pathological conditions may be responsible, including hypertension, hyperglycemia and dyslipidemia (5, 22-25). Notably, it has been reported that microalbuminuria predicts vascular disease and that proteinuria may be a key marker for CVD and even death (5). Ostensibly, as nephropathy progresses, secondary metabolic disturbances may accelerate atherosclerosis and the onset of CVD (2). As these vascular changes emerge early in individuals with T1D, it is important to identify modifiable risk factors, such as dietary habits, to ameliorate the CVD risk profile in this population (26).

T1D management approaches are individualized and focus largely on monitoring carbohydrate intake and integrating an insulin regimen to achieve glycemic control (27). Generally, individuals with T1D are encouraged to consume a balanced diet with an emphasis on produce, lean meats and whole grains and a limited intake of simple sugars and saturated fats (28-31). The literature on dietary egg consumption in T1D is limited. Existing studies in this population address dietary egg intake as part of an assessment on dietary patterns as a whole (26, 30, 32, 33). To the best of our knowledge, this is the first study investigating dietary whole eggs as a nephroprotective food and a source of vitamin D in a T1D model.

Nephropathy is a major factor implicated in vitamin D deficiency in the diabetic population. Because 25D circulates bound to DBP, the 25D-DBP complex must be internalized by the kidney for subsequent release of 25D and activation to 1,25D. Thus, as diabetic nephropathy progresses there is an increase in the filtration of the 25D-DBP complex resulting in excessive urinary loss of 25D and diminished serum 25D concentrations. We previously reported that a whole egg-containing diet is a highly effective strategy for maintaining serum 25D concentrations in T2D (14). On the basis of those observations, we hypothesized that a whole egg-based diet would have a similar impact on serum 25D concentrations in a T1D model. Although vitamin D deficiency did not develop in this acute model (i.e., 1 week) of T1D, it is important to note that hypovitaminosis D is highly prevalent among individuals with T1D (9, 34, 35). Our findings show that T1D rats fed whole egg had significantly higher serum 25D concentrations than casein-fed rats, regardless of diabetes status. Because whole egg consumption resulted in urinary concentrations of 25D and DBP that did not differ from non-diabetic rats, or T1D rats fed casein, we expect that future studies with a larger sample size may result in more robust differences in urinary vitamin D loss. Furthermore, studies with a longer treatment period following the induction of T1D may allow for sufficient time for vitamin D deficiency to develop; thus, we postulate that for a longer study period, whole egg consumption would increase circulating 25D in T1D rats due to the rich vitamin D content of dietary egg combined with the observed nephroprotective effects.

The number of individuals with T1D is growing due to the increasing number of new-onset cases in adults and those who were diagnosed in childhood with T1D and are living longer (36, 37). As the prevalence increases, the need for treatment modalities to prevent or

slow progression of T1D-related complications is critical. Our previous studies demonstrated that dietary resistant starch was nephroprotective in T1D and T2D rats (12, 13). The present study suggests that whole egg consumption may protect against polyuria, urinary protein and vitamin D losses, and renal hypertrophy in T1D. Future studies will investigate a diet containing both whole egg and resistant starch to determine whether a combination of these two strategies results in an additive effect with respect to renal protection and vitamin D balance. Understanding the mechanism underlying the nephroprotective effect of dietary whole egg will be a focus of future work as well.

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C.J.S and S.K.J. performed all aspects of animal maintenance, preparation of experimental diets, and laboratory experiments as well as drafted the original version of this manuscript. K.L.S. and M.J.R. assisted with the study design. All authors read and approved the final manuscript.

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Tables and Figures

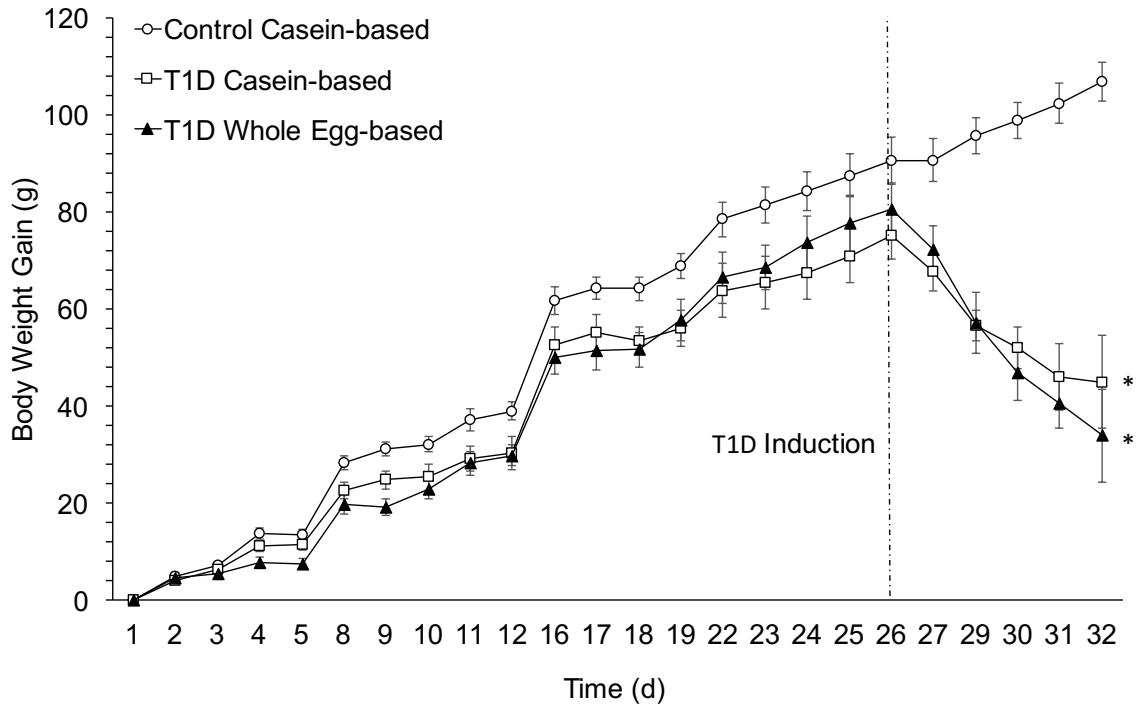


Figure 1. Cumulative body weight gain over the 4-week experimental period. Sprague-Dawley rats were fed a casein-based or a whole egg-based diet for 32 days and type 1 diabetes (T1D) was induced on day 26 in two-thirds of the rats via streptozotocin. Body weights were recorded daily and reported as cumulative body weight gain from day 0. Data are expressed as mean values \pm SEMs; $n = 5-6$. Values with an asterisk differ from the control ($P < 0.05$).

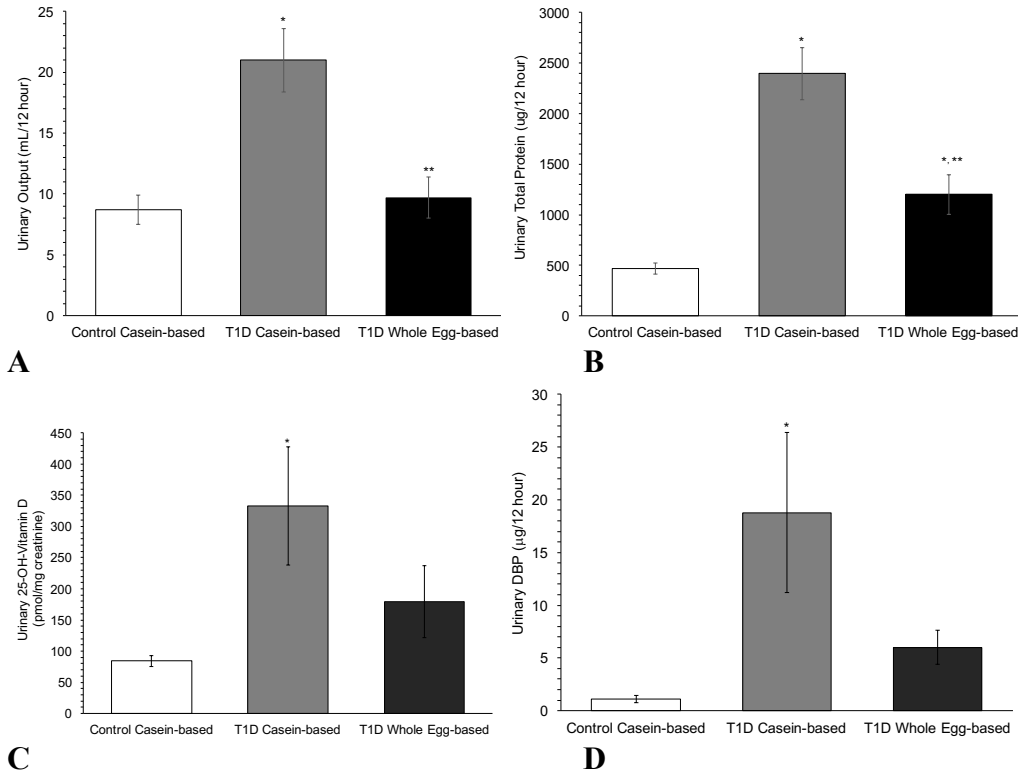


Figure 2. Whole egg consumption attenuated urinary markers of nephropathy. Total urinary output (A), urinary total protein (B) 25-hydroxycholecalciferol (25D) and (C) vitamin D binding protein (DBP) concentrations (D) of male Sprague-Dawley rats following 32 days dietary treatment with a casein- or whole egg-based diet. Type 1 diabetes (T1D) was induced on day 26 in two-thirds of the rats via streptozotocin. Data are expressed as mean values \pm SEMs; $n = 4-5$. Bars with an asterisk differ from the control ($P < 0.05$). Significant differences between T1D rats following a Student's t-test are indicated by two asterisks ($P < 0.05$).

Table 1. Composition of casein- and whole egg-based diets fed to male Sprague-Dawley control and type 1 diabetic rats for 32 days.

ingredient ¹	casein-based diet	whole egg-based diet
		g/kg
casein	200	0
whole egg ^{2,3}	0	408
cornstarch	437	392
glucose	150	150
mineral mix	35	35
vitamin mix ³	10	10
corn oil	163	0
choline bitartrate	2	2
L-methionine	3	3

¹All ingredients were purchased from Envigo (Madison, WI, USA) with the exception of dried whole egg (Rose Acre Farms, Guthrie Center, IA, USA) as well as L-methionine and choline bitartrate (Sigma Aldrich, Milwaukee, WI, USA).

²Total protein and lipid content provided by 408 g of dried whole egg is 49% (200 g) and 40% (163 g), respectively.

³Total cholecalciferol provided by casein-based and whole egg-based diets are 25 and 37.6 µg/kg diet, respectively.

Table 2. Final body weight, blood glucose and serum 25-hydroxycholecalciferol (25D) concentrations of control and type 1 diabetic (T1D) male Sprague-Dawley rats fed a casein-based diet or whole egg-based diet for 32 days (1).

	control casein-based	T1D casein-based	T1D whole egg-based
final body weight (g)	404 ± 9	326 ± 11*	320 ± 15*
blood glucose (mg/dL)	258 ± 41	637 ± 76*	548 ± 133*
serum 25D (nmol/L)	30 ± 1	33 ± 2	54 ± 3*

¹Data are expressed as mean values ± SEMs; n=6. Data with an asterisk differ from control ($P < 0.05$).

Table 3. Relative kidney weight and urinary creatinine concentrations of control and type 1 diabetic (T1D) male Sprague-Dawley rats fed a casein-based diet or whole egg-based diet for 32 days (1, 2).

	control casein-based	T1D casein-based	T1D whole egg-based
kidney weight (% body weight)	0.633 ± 0.025	1.009 ± 0.0327*	0.836 ± 0.0286*, **
urinary creatinine (mg/12 hrs)	3.3 ± 0.2	2.4 ± 0.5	2.2 ± 0.8

¹Data are expressed as mean values ± SEMs; n=6. Data with an asterisk differ from control ($P < 0.05$).

²Significant differences between T1D rats following a Student's *t*-test are indicated by two asterisks ($P < 0.05$).

CHAPTER 6. WHOLE EGG CONSUMPTION INCREASES SERUM 25-HYDROXYVITAMIN D3 CONCENTRATIONS IN RATS WITH DEXTRAN SULFATE SODIUM-INDUCED COLITIS

A manuscript prepared for submission to the *Journal of Agriculture and Food*

Chemistry

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Abstract

Vitamin D deficiency is prevalent among individuals with inflammatory bowel disease (IBD) and may contribute to IBD-associated complications. We previously demonstrated that a whole egg-containing diet maintained vitamin D balance in type 1 and type 2 diabetic rats. The goal of the present study was to investigate the impact of whole egg consumption in dextran sulfate sodium (DSS)-induced colitis. In an initial dose response study, Sprague Dawley rats were maintained on a casein-based diet for 5 weeks and randomly assigned to 0, 3, 4 or 5% DSS-treated drinking water for the final 7 days. Serum 25D concentrations exhibited a dose-response decrease with respect to increasing DSS concentrations. In a follow-up study, Sprague Dawley rats were randomly assigned to a casein-based diet, a dried whole egg-based diet, or a casein-based diet containing supplemental cholecalciferol provided at the same level of cholecalciferol supplied by the whole egg-based diet, for 5 weeks. Half of the rats in each group were given 3.5% DSS-treated drinking water for the final 7 days of the study. Rats fed a whole egg-based diet exhibited increased serum 25D concentrations that were significantly higher than rats in either of the other dietary intervention groups, regardless of colitis status. These data suggest

that whole egg consumption may be more effective than supplemental cholecalciferol at increasing circulating 25D concentrations in experimental colitis.

Introduction

In 2015 approximately one-third of the adult population in the U.S. had been diagnosed with inflammatory bowel disease (IBD), a chronic, relapsing inflammatory condition of the gastrointestinal (GI) tract that is classified into two types: Crohn's disease (CD) and ulcerative colitis (UC) (1). CD may affect any portion of the GI tract, from the mouth to perianal area. It is characterized by a discontinuous and ulcerous transmural inflammation that extends through the intestinal wall from mucosa to serosa. Symptoms of CD include abdominal pain, fever, bloody or non-bloody diarrhea, and weight loss (2). On the contrary, UC is characterized by superficial mucosal inflammation and only affects the colon. Symptoms include rectal bleeding, diarrhea as well as abdominal pain (3). The exact cause of IBD remains unclear; however, it is thought to be due to a combination of a person's genetics, microbiome, and the environment that result in an excessive and inappropriate immune response against commensal flora in genetically susceptible individuals (4).

Vitamin D has been recognized as an environmental factor that may influence the pathogenesis and progression of IBD (5-8). The prevalence of hypovitaminosis D is up to 75% in those with CD and up to 60% in individuals with UC (9). While the role of vitamin D signaling in the gut has not been fully elucidated, vitamin D is implicated in preserving mucosal integrity; thus, its deficiency may compromise or disrupt intestinal barrier function and lead to a local immune response (9). Factors accounting for vitamin D deficiency in IBD include inadequate daily intake, inflammation and glucocorticoid therapies. Importantly,

diarrhea, malabsorption, and GI bleed are common features of IBD; thus, nutritional deficiencies are common in individuals with UC and CD (5).

Despite the wealth of research on the influence of diet and IBD, there are no standardized therapeutic dietary guidelines for individuals with UC and CD. Dietary recommendations for individuals with IBD are personalized and focus on restoring nutritional status and alleviating symptoms 10. Whole eggs are a rich dietary source of vitamin D, including both vitamin D₃ and 25D, specifically found in the yolk (11). We recently reported that a whole egg-containing diet is effective at maintaining vitamin D status in type 1 and type 2 diabetic rats, diseases characterized by vitamin D deficiency (12-14). The literature remains inconclusive with respect to the inclusion of whole eggs in the diet for individuals with IBD. Although some studies conclude egg consumption is well tolerated, others have deemed eggs a sensitive food item for those with IBD (15-17). To date, there are no studies examining the consumption of whole egg specifically in individuals with IBD either as a dietary treatment and/or as a significant food source of vitamin D. Moreover, the capacity of a food item to assist with maintenance of vitamin D may be a viable avenue for attenuation of IBD symptoms. Thus, the goal of this study was to characterize vitamin D deficiency as a function of experimental colitis and compare vitamin D in a whole egg-based diet to a diet containing supplemental vitamin D₃ (i.e., cholecalciferol), with respect to maintaining vitamin D homeostasis in rats with dextran sulfate sodium (DSS)-induced colitis.

Materials and Methods

Chemicals. All chemicals were of analytical grade and purchased from commercial suppliers. Authenticity of all kits for use on rodent biological samples has been verified by the manufacturer.

Animals and Diets. All animal studies were approved by the Institutional Animal Care and Use Committee at Iowa State University and were performed according to the Iowa State University Laboratory Animal Resources Guidelines. In an initial dose response study, 5-week-old Sprague Dawley rats (N=24) were randomly assigned to four treatment groups of DSS- treated (MP Biomedicals, Santa Ana, CA, USA) drinking water (% w/v): 0 (n=6), 3 (n=6), 4 (n=6), and 5% (n=6). All rats were maintained on a modified AIN93 casein-based diet for 5 weeks. DSS-treated drinking water replaced tap water for the final 7 days of the study period. In a follow-up study, male Sprague Dawley rats (N=36) were obtained at 5 weeks of age (Envigo, Madison, WI, USA). Rats were housed individually in ventilated cages (Innovive, San Diego, CA, USA) with a 12-hour light-dark cycle in a temperature-controlled room. All diets were formulated and pelleted by Research Diets, Inc (New Brunswick, NJ, USA). Dried whole egg was purchased from Rose Acre Farms (Seymour, IN, USA) and sent to Research Diets, Inc. for diet formulation. Following one week of acclimation to a semi purified diet (AIN-93), rats were randomly assigned to 1 of 3 dietary treatment groups (Table 1): a casein-based diet, a dried whole egg-based diet, or a casein-based diet containing supplemental cholecalciferol provided at the same level of cholecalciferol supplied by the whole egg diet (37.6 $\mu\text{g}/\text{kg}$ diet). Vitamin mix in all diets provided 25 μg vitamin D/kg diet. The whole egg-based diet contained an additional 12.6 μg cholecalciferol/kg diet equating to 37.6 μg cholecalciferol/kg of the whole egg-based diet. This level was matched in the casein-based diet containing supplemental cholecalciferol with the addition of 12.6 μg cholecalciferol. All diets provided protein at 20% (w/w) and were

matched for lipid content (18.3%) via the addition of corn oil to the casein-based diets, accounting for the additional lipid provided by the dried whole egg.

For the final 7 days of the study, half of the rats in each diet group received 3.5% DSS drinking water. Based on the results of the DSS dose response study, 3.5% was determined to be the optimal concentration to induce experimental colitis and minimize premature rodent death. Rats were given ad libitum access to food and water for the 5-week period. For all rats in the dietary intervention study, disease index activity (DIA) scores were recorded daily throughout the DSS period. DIA criteria included percent weight loss, stool consistency, severity of rectal bleeding and color of rectal blood (18, 19). Each criterion was measured on a scale of 0-4 (Table 2) and then averaged together. For all studies, rats were placed in metabolism cages for 12 hours prior to sacrifice, to ensure that they were in a fasted state and for the collection of urine. Rats were anesthetized via a single intraperitoneal injection of ketamine:xylazine (90:10 mg/kg body weight) and whole blood was collected by cardiac puncture. Liver, kidney and colon were removed and weighed, colonic length was measured, and tissues were immediately freeze-clamped in liquid nitrogen for storage at -80°C.

Biochemical Assessment. Analysis of urinary creatinine was measured using a commercially available colorimetric kit (Cayman Chemical, Ann Arbor, MI, USA). Urinary total protein concentrations were measured using a bicinchoninic acid colorimetric assay (Thermo Fisher Scientific, Waltham, MA, USA). Serum and urinary 25D concentrations as well as urinary DBP were analyzed using commercial ELISA kits (Immunodiagnostic Systems,

Gaithersburg, MD, USA, and Life Diagnostics, West Chester, PA, USA respectively), all as previously described (12, 14, 20).

Statistical Analysis. All data were analyzed using JMP Version 10.02 (SAS Institute Inc., Cary, NC, USA) software. Mean values were evaluated for statistically significant differences ($P < 0.05$) using a one-way or two-way (genotype x diet) ANOVA followed by the Tukey's Honest Significant Difference (HSD) post hoc test for multiple comparisons.

Results

Body Weight, Food Intake and Disease Activity Index Scores. Cumulative body weight gain, in rats given 0, 3, 4, or 5% DSS-treated drinking water for 7 days, over the 5-week experimental period is shown in Figure 1A. Rats in the 4 and 5% DSS groups exhibited a 45% reduction in body weight gain compared to rats that consumed 0 and 3% DSS drinking water ($P < 0.0001$). As expected, final body weight was lowest in the 5% DSS group ($P < 0.0001$), but statistically the same when compared to rats in the 4% group ($P = 0.86$) (Figure 1B). When comparing dietary treatment interventions in a follow-up study, diet was without effect on body weight gain patterns, regardless of whether or not rats received DSS drinking water (Figure 2A). No differences were observed in final body weight or food intake patterns across dietary groups throughout the 5-week study period, regardless of colitis status (data not shown). Likewise, DIA scores measured in rats with experimental colitis did not differ across dietary treatment groups, averaging 2.4 out of a 4.0 scale (Figure 2B).

Serum Parameters and Relative Tissue Weights. Serum 25D concentrations exhibited a dose-response decrease with respect to increasing DSS concentrations. Serum 25D

concentrations were approximately 83% higher in control rats given 0% DSS drinking water ($P < 0.0001$) compared to rats in the 4 and 5% DSS groups, whose serum 25D concentrations were the same (Figure 3A). Rats in the 3% DSS group exhibited a 17% decrease ($P = 0.001$) in serum 25 concentrations compared to rats in the 0% DSS group, and serum 25D concentrations were 52% higher ($P < 0.0001$) compared to rats given 4 and 5% DSS-treated drinking water (Figure 3A). When comparing dietary treatment effects, rats that were fed the whole egg-based-diet had increased serum 25D concentrations that were approximately 78% higher ($P < 0.0005$) than rats in either of the casein-based diet groups, regardless of whether rats consumed the 3.5% DSS-treated water (Figure 3B).

Relative colon weight is presented in Figure 4. When comparing DSS-dose, relative colon weight was the same across the 0 and 3% DSS groups ($P = 0.77$) as well as across the 4 and 5% groups ($P = 0.80$) (Figure 4A). Relative colon weight was highest in the 4% group compared to 0% ($P = 0.0020$), and 3% DSS ($P = 0.0136$). No differences were observed between the 3% and 5% DSS groups ($P = 0.13$) (Figure 4A). In the follow-up dietary intervention study, relative colon weights were significantly lower in control rats that received tap water compared to 3.5% DSS-treated water ($P < 0.0001$) (Figure 4B). Rats with colitis fed a whole egg-based diet exhibited relative colon weights that were the same as healthy rats ($P = 0.13$); however, not different from casein-fed rats with experimental colitis ($P = 0.54$) (Figure 4B).

Relative kidney weights exhibited an increasing trend with increasing DSS concentrations (Figure 5A). Compared to 0% DSS, relative weights of kidneys were 30, 39, and 55% higher in the 3, 4 and 5% groups, respectively ($P = 0.056$, $P = 0.014$, $P = 0.002$, respectively). Diet was without effect on relative kidney weights within rats fed the casein-

based diet containing supplemental cholecalciferol and the whole egg-based diet, compared to rats fed the casein-based diet ($P = 0.11$); however, when analyzed using a Student's t-test, rats with DSS-induced colitis exhibited significantly higher relative kidney weights compared to control, healthy rats ($P = 0.005$) (Figure 5B).

Urinary Parameters. DSS dose was without effect on urine output, urinary total protein, creatinine, 25D and DBP in rats given 0, 3, 4 and 5% DSS-treated drinking water for 7 days (Table 3). Similarly, no differences in urinary parameters were exhibited across dietary treatment groups (Table 4). Notably, within the dietary intervention study, rats with DSS-induced colitis exhibited significantly less urine output ($P = 0.001$), greater total protein excretion ($P = 0.009$) and increased urinary loss of DBP ($P = 0.002$) compared to healthy control rats (Table 4).

Discussion

We have previously shown that a dried whole egg-based diet is a highly effective strategy for maintaining serum 25D concentrations in rats with T1D and T2D, conditions characterized by vitamin D deficiency (12-14). The present study demonstrated that 4 and 5% DSS-treated drinking water leads to a vitamin D-deficient condition in an experimental model of colitis in Sprague Dawley rats. Furthermore, we showed that inclusion of dried whole egg, in the diet of rats with and without DSS-induced colitis, was more effective than an equivalent amount of supplemental cholecalciferol at increasing serum 25D concentrations. Serum 25D concentrations were significantly higher in rats fed a whole egg-based diet compared to rats fed a casein-based diet containing supplemental cholecalciferol, regardless of colitis status. Animal-based foods, including eggs, are known to contain

varying amounts of vitamin D3 and 25D (21). In fact, it has been reported that eggs contain higher concentrations of 25D compared to other animal-based foods (11, 22). Recent evidence suggests that 25D is approximately five times more potent than vitamin D3 in raising circulating 25D concentrations (11, 23, 24). Thus, the contrast in serum 25D concentrations between rats fed a whole egg-based diet versus either of the casein-based diets may be due to this increased potency of 25D.

Apart from vitamin D, eggs contain a variety of other nutrients, particularly when compared to other animal products. They have been promoted for their high-quality protein, contributing to satiety, as well as for their high nutrient density-to-energy ratio (25, 26). One egg equates to 3.6% of total calories while at the same time providing a number of other nutrients in excess of its caloric contributions including iron, vitamin B12, folate, riboflavin, choline, and vitamin A (27). Malnutrition is a well-known complication of IBD. Based on BMI analysis, the prevalence of malnutrition appears to be higher in CD compared to UC, although there are several reports of a similar prevalence in both conditions (28-31). In pediatric patients with IBD, malnutrition is a primary cause of growth retardation and a 20% delay in puberty onset (32). The impairment of nutritional status in IBD is multifactorial. The leading causes include suboptimal energy intake, malabsorption, enteric nutrient loss, increased basal energy expenditure, and medications (9). In clinical settings, individuals with UC and CD are commonly found to be underweight, with several nutritional deficiencies, alterations of anthropometric parameters, low bone mineral density and a reduction in fat and muscle mass (29, 30, 33, 34).

Because there are no specific dietary restrictions for individuals with IBD, with respect to management of relapse or prevention during remission, egg consumption may be

an optimal source of nutrients, specifically vitamin D, to support overall health in UC and CD. In a study assessing the efficacy of the Anti-Inflammatory Diet (IBD-AID) in the co-management of IBD, developed by Olendzki and colleagues (17), the nutritional regimen restricted the intake of specific carbohydrates, included pre- and probiotic foods, and modified dietary fatty acids (17). The diet supported the intake of eggs as a source of omega-3 fatty acids, though the investigators did not report quantity consumed by participants. After following the IBD-AID for four weeks, all 11 patients reported a reduction in symptom severity and were able to discontinue at least one of their IBD medications (17). Similarly, Chiba et al (35) found that a semi-vegetarian diet, with the inclusion of egg consumption, was highly effective in preventing relapse in CD (35). These studies demonstrate the potential for adjunct dietary therapy, with eggs as part of a balanced intake, for the treatment of IBD. On the contrary, a prospective cohort study investigating the dietary factors associated with an increased risk of relapse of UC suggested that high intake of meat and meat products, including eggs, predicted an increased likelihood of relapse (16). It was suggested that the sulfur compounds within these foods, including eggs, may mediate the likelihood of relapse, but further studies are required to determine if reducing their intake would reduce relapse frequency (16, 36). In another study assessing the role of macronutrients and the etiology of IBD among middle-aged French women, high consumption of meat or fish, but not of eggs or dairy products, was associated with IBD risk (37).

Individuals with IBD often develop one or more extraintestinal manifestations (EIM) during the course of disease (38-40). The prevalence of EIM varies from 6-46% in human cases (39). Though the etiology remains unclear, EIM have been attributed to genetic factors,

circulating bacterial endotoxins, infectious agents and the unwanted presence of antigen-antibody complexes in the affected tissue (39). Impaired renal function is considered an EIM of IBD. It has been described in UC and CD (41-43) and also reported in animal models of IBD (44, 45). Renal diseases in IBD include nephrolithiasis, tubulointerstitial nephritis, glomerulonephritis and amyloidosis (41). In the present study, our follow up investigation demonstrated that rats with DSS-induced colitis exhibited a decrease in urine output, increased proteinuria, increased urinary excretion of DBP and significantly higher relative kidney weights. These findings may indicate the presence of renal hypertrophy and EIM of experimental colitis in the kidneys. In contrast to results from our T1D study (14), in which whole egg consumption was found to be nephroprotective in T1D rats, diet was without effect on urinary parameters in rats with experimental colitis. Of note, given the extent of diarrhea and blood loss in colitic rats, we cannot rule out the possibility that dehydration contributed to the decrease in urine output and increased urinary protein loss observed in the present study. Additional analysis of kidney tissue is necessary to rule out hypertrophy and tissue injury.

There is a high prevalence of vitamin D deficiency among individuals with UC and CD, owing to secondary disease complications including inflammation, malabsorption, diarrhea and GI bleed (9). Furthermore, a vitamin D deficient condition may exacerbate these symptoms (5). Although vitamin D deficiency did not develop in rats given 3.5% DSS in the follow-up experiment presented in this study, we expect that prolonged exposure to 3.5% DSS, or a higher DSS concentration for 7 days, would lead to hypovitaminosis D in colitic rats, as was demonstrated in the first experiment among rats in the 4 and 5% DSS groups. Regardless, our studies highly suggest that a whole egg-containing diet has the capacity to

increase circulating 25D concentrations in experimental colitis and may be more effective than supplemental cholecalciferol. In addition to improving the experimental model, further studies will also focus on the dose response of dried whole egg to determine the minimal amount required to maintain vitamin D homeostasis.

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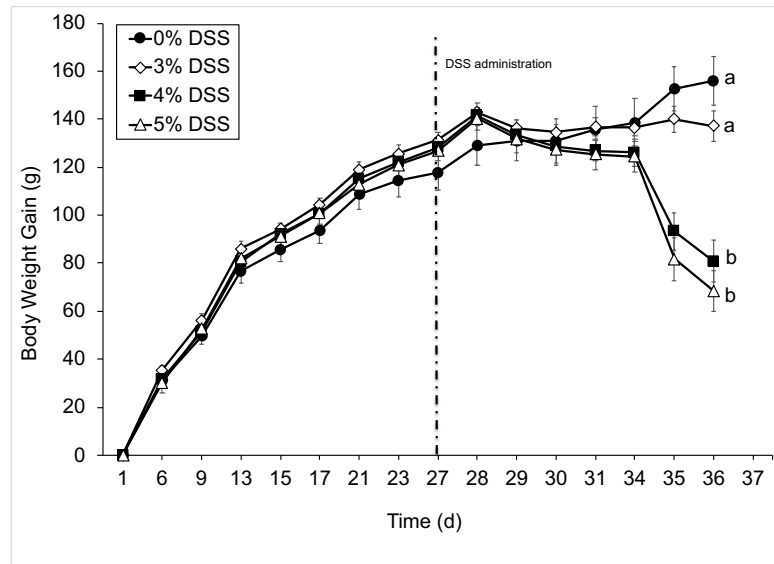
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Tables and Figures

A



B

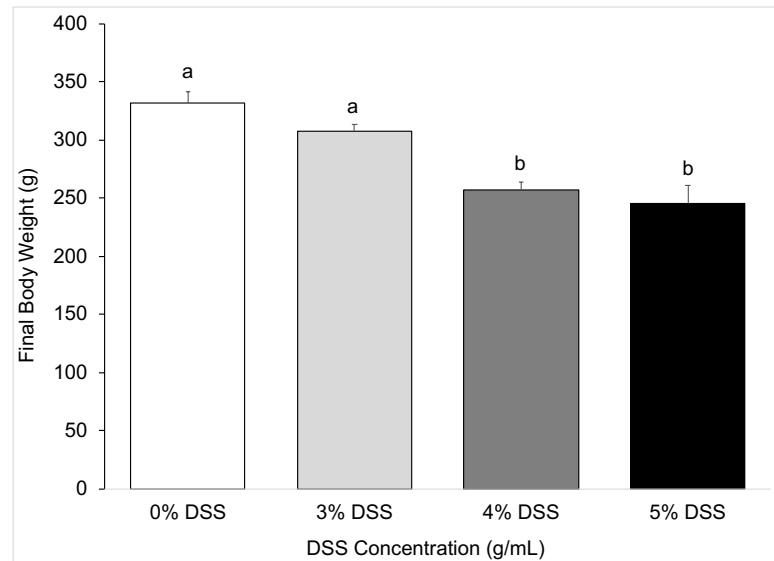
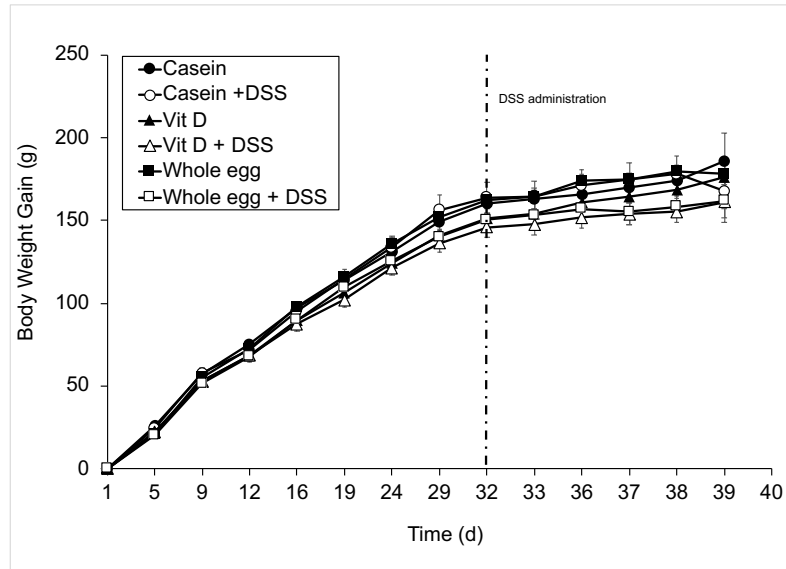


Figure 1. Cumulative body weight gain and final body weight over the 5-week experimental period. Sprague Dawley rats were maintained on a casein-based diet for 5 weeks. 0, 3, 4 and 5% dextran sulfate sodium-treated drinking water replaced tap water for the final 7 days of the study. Body weights were recorded daily and reported as cumulative body weight gain from day 0 (A) and body weight in grams on the final study day (B). Data are expressed as mean values \pm SEMs; $n = 4-6$. Mean values without a common letter are statistically significant ($P < 0.05$).

A



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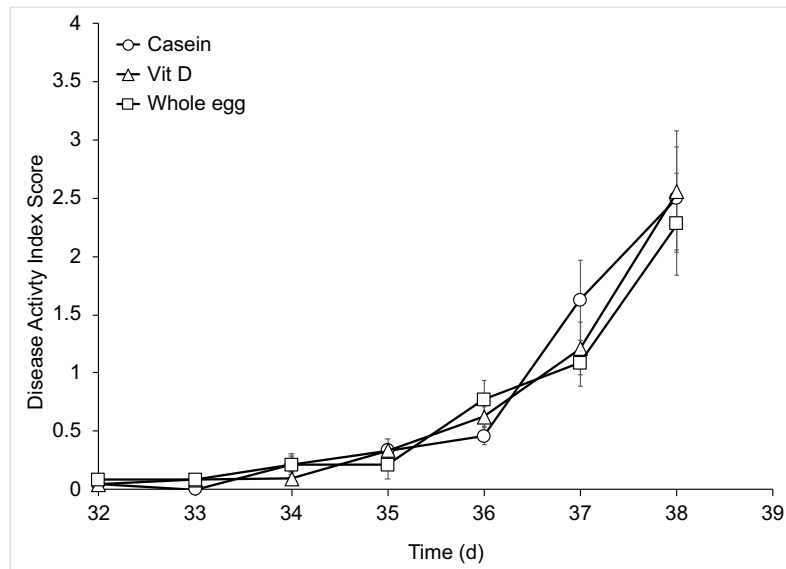
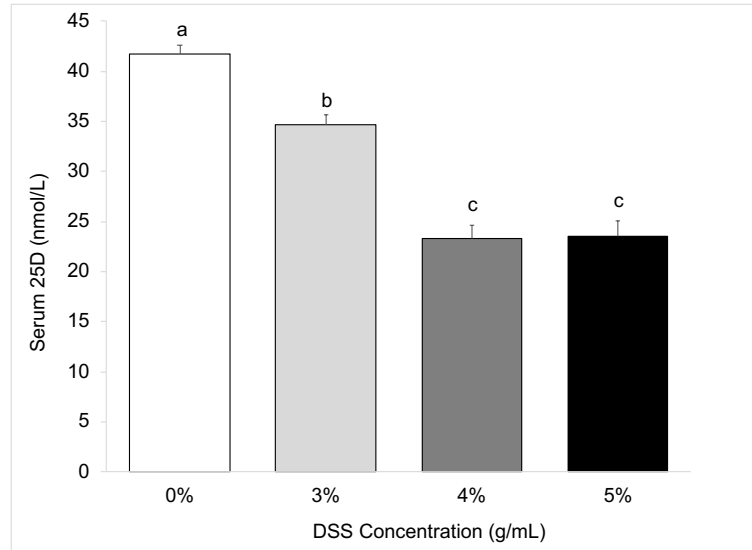


Figure 2. Dietary intervention was without effect on cumulative body weight gain and disease activity index (DAI) scores. Sprague Dawley rats were maintained on a casein-, supplemental cholecalciferol- or a whole egg- based diet for 5 weeks. 3.5% dextran sulfate sodium-treated drinking water replaced tap water for the final 7 days of the study. Body weights were recorded daily and reported as cumulative body weight gain from day 0 (A). DIA criteria included percent weight loss, stool consistency, severity of rectal bleeding and color of rectal blood. Each criterion was measured individually on a scale of 0-4 and reported as an average per group (B). Data are expressed as mean values \pm SEMs; $n = 6$. Mean values without a common letter are statistically significant ($P < 0.05$).

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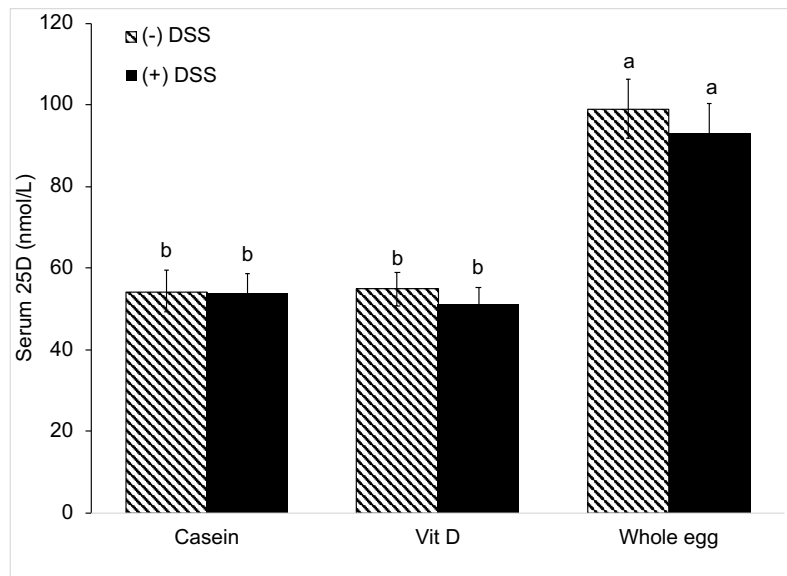
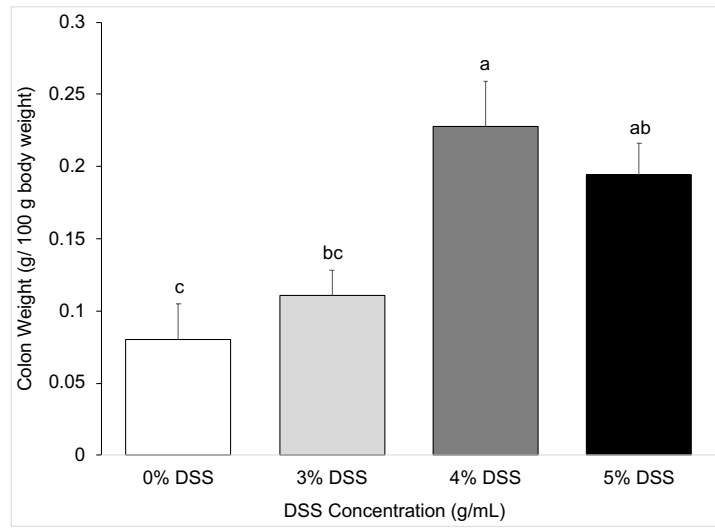


Figure 3. Serum 25-hydroxyvitamin D (25D) concentrations decreased with increasing dextran sulfate sodium concentrations and were significantly higher in rats fed a whole egg-based diet. Sprague Dawley rats were maintained on a casein-based diet for 5 weeks. 0, 3, 4 and 5% dextran sulfate sodium-treated drinking water replaced tap water for the final 7 days of the study (A). In a follow-up study, Sprague Dawley rats were fed either a casein-, supplemental cholecalciferol- or a whole egg-based diet for 5 weeks. 3.5% dextran sulfate sodium-treated drinking water replaced tap water for the final 7 days of the study (B). For all studies, at the end of the treatment period rats were anesthetized, and whole blood was collected by cardiac puncture. 25D concentrations were measured using a commercial enzyme immunoassay kit. Data are expressed as mean values \pm SEMs; $n=4-6$. Mean values without a common letter are statistically significant ($P < 0.05$).

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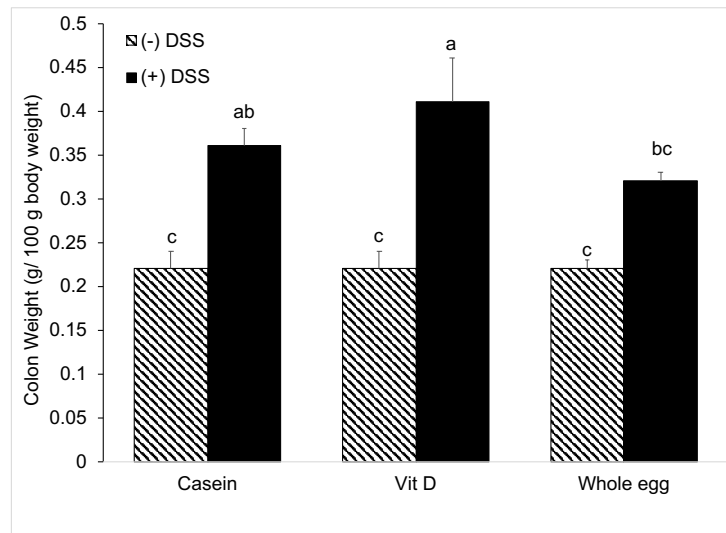
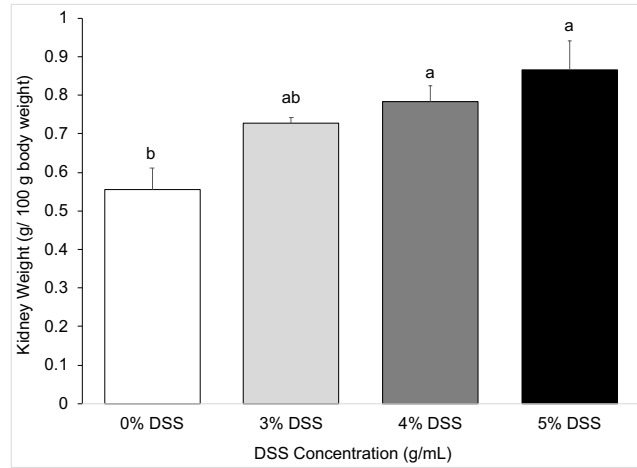


Figure 4. Relative colon weight was highest in rats given 4 and 5% dextran sulfate sodium-treated drinking water, and the same between healthy rats and rats fed a whole egg-based diet. Sprague Dawley rats were maintained on a casein-based diet for 5 weeks. 0, 3, 4 and 5% dextran sulfate sodium-treated drinking water replaced tap water for the final 7 days of the study (A). In a follow-up study, Sprague Dawley rats were fed either a casein-, supplemental cholecalciferol- or a whole egg- based diet for 5 weeks. 3.5% dextran sulfate sodium-treated drinking water replaced tap water for the final 7 days of the study (B). For all studies, at the end of the treatment period rats were anesthetized, and whole blood was collected by cardiac puncture. Colons were removed, weighed and reported as grams per 100 grams of body weight. Data are expressed as mean values \pm SEMs; $n=4-6$. Mean values without a common letter are statistically significant ($P < 0.05$).

A



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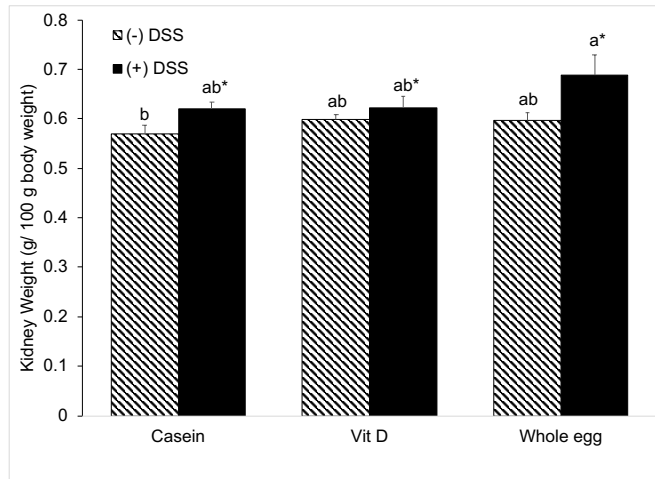


Figure 5. Relative kidney weight exhibited a dose-dependent response to increasing dextran sulfate sodium (DSS) concentrations in rats given 0, 3, 4 and 5% DSS-treated drinking water, and in a follow-up study remained significantly higher in rats given 3.5% DSS compared to rats given normal tap water. Sprague Dawley rats were maintained on a casein-based diet for 5 weeks. 0, 3, 4 and 5% dextran sulfate sodium-treated drinking water replaced tap water for the final 7 days of the study (A). In a follow-up study, Sprague Dawley rats were fed either a casein-, supplemental cholecalciferol- or a whole egg- based diet for 5 weeks. 3.5% dextran sulfate sodium-treated drinking water replaced tap water for the final 7 days of the study (B). For all studies, at the end of the treatment period rats were anesthetized, and whole blood was collected by cardiac puncture. Kidneys were removed, weighed and reported as grams per 100 grams of body weight. Data are expressed as mean values \pm SEMs; $n=4-6$. Mean values without a common letter are statistically significant ($P < 0.05$).

Table 1. Composition of the casein-based diet, casein-based diet including supplemental cholecalciferol (vit D) and whole egg-based diet fed to control and experimental colitis Sprague Dawley rats for 5 weeks.

ingredient ¹	casein	vit D g/kg	whole egg
casein (vitamin-free)	200	200	0
dried whole egg ^{2,3,4}	0	0	435
cornstarch	417	417	365
glucose monohydrate	150	150	150
mineral mix (AIN 93)	35	35	35
vitamin mix (AIN 93)	10	10	10
cholecalciferol, 100,000 IU/g	0	0.00504	0
biotin, 1%	0	0	0.4
corn oil	183	183	0
choline bitartrate	2	2	2
L-methionine	3	3	3

¹All diets were formulated by and purchased from Research Diets Inc (New Brunswick, NJ, USA).

²Whole egg was purchased from Rose Acre Farms (Seymour, IN, USA) and sent to Research Diets Inc. for diet formulation.

³Total protein and lipid content provided by 435 g of whole egg were 46% (200 g) and 42% (183 g), respectively.

⁴Total cholecalciferol provided by the casein-based diet, casein-based diet including supplemental cholecalciferol and whole egg-based diet were 25, 37.6 and 37.6 µg/kg diet, respectively.

Table 2. Disease Activity Index (DAI) Criteria¹

score	% weight loss	stool consistency	rectal bleeding	color of rectal blood
0	none	normal	negative	normal
1	1-5		spotting	red
2	6-10	loose	gross bleeding	dark
3	11-15		gross bleeding > 1 day	dark red
4	>15	diarrhea	gross bleeding > 2 days	black

¹Adapted from Rath et al, 2012 (18) and Kim et al, 2012 (19)

Table 3. Urinary parameters of male Sprague Dawley rats given 0, 3, 4 or 5% dextran sulfate sodium-treated drinking water for 7 days¹

	0% DSS	3% DSS	4% DSS	5% DSS
urine output (mL/12hr)	8.2 ± 1.5	5.8 ± 1.3	13 ± 4.0	7.3 ± 1.7
urinary total protein (mg/ 12 hr)	1.3 ± 0.4	2.2 ± 0.6	0.8 ± 0.4	1.2 ± 0.5
urinary creatinine (mg/12 hr)	1.9 ± 0.2	2.1 ± 0.1	1.9 ± 0.1	1.9 ± 0.3
urinary 25D (pmol/mg)	72 ± 11	61 ± 15	137 ± 55	88 ± 28
urinary DBP (µg/ 12 hr)	0.05 ± 0.01	0.6 ± 0.3	0.3 ± 0.1	0.4 ± 0.2

¹Data are expressed as mean values ± SEMs; n=4-6. Mean values within a row without a common letter are statistically significant ($P < 0.05$).

Table 4. Urinary parameters of control and colitic male Sprague Dawley rats fed a casein-based diet, casein-based diet including supplemental cholecalciferol (vit D) and whole egg-based diet fed to control and DSS rats for 5 weeks^{1,2}

	casein		vit D		whole egg	
	(-) DSS	(+) DSS	(-) DSS	(+) DSS	(-) DSS	(+) DSS
urine output (mL/12 hr)	9.2 ± 3.5	1.2 ± 0.2*	8.4 ± 2.1	0.9 ± 0.3*	7.8 ± 2.8	1.2 ± 0.4*
urinary total protein (mg/ 12 hr)	2.7 ± 1.5	40 ± 6*	3.8 ± 3	179 ± 92*	2.2 ± 0.8	91 ± 37*
urinary creatinine (mg/12 hr)	5.7 ± 1.1	5.3 ± 0.6	5.3 ± 0.3	3.8 ± 0.8	4.1 ± 1.4	2.4 ± 0.8
urinary 25D (pmol/mg)	44 ± 11	8.5 ± 0.8	98 ± 36	16 ± 5.3	131 ± 63	89 ± 45
urinary DBP (µg/ 12 hr)	2.0 ± 1.1	12 ± 1.7*	1.2 ± 1.0	38 ± 14*	2.0 ± 1.0	37 ± 13*

¹Data are expressed as mean values ± SEMs; n=6. Mean values within a row without a common letter are statistically significant ($P < 0.05$).

²Significant differences between (-) DSS and (+) DSS rats following a Student's *t*-test are indicated by an asterisk ($P < 0.05$)

CHAPTER 7. GENERAL CONCLUSIONS

Overall Summary and Conclusions

Accumulating evidence from observational data and clinical trials have demonstrated an association between hypovitaminosis D and a variety of chronic diseases, including diabetes and inflammatory bowel disease (IBD) (1). Declining trends in circulating 25D concentrations may be the result of a combination of insufficient dietary intake and limited sunlight exposure. Individuals with diabetes and IBD are at an increased risk for vitamin D deficiency due to renal insufficiency and malabsorption, respectively. Moreover, vitamin D deficiency may exacerbate the progression of secondary disease complications (2, 3). Whole eggs contain high quality protein and provide several nutrients in excess of its caloric content. Importantly, they are one of few food sources naturally rich in vitamin D3 and 25D (4). The studies presented in this dissertation have demonstrated that dietary whole egg consumption is an effective means of increasing serum 25D concentrations in streptozotocin-induced type 1 diabetes (T1D), a well-established model of type 2 diabetes (T2D) using Zucker Diabetic Fatty (ZDF) rats, and dextran sulfate sodium (DSS)-induced colitis.

With respect to diabetes, our lab previously determined that the loss of vitamin D in the urine is a result of compromised renal function in ZDF rats (5), and a dietary intervention with resistant starch was protective against diabetic nephropathy in T1D rats (6). Results from the T1D study presented in this dissertation demonstrate that a dietary intervention incorporating dried whole eggs in the diet of streptozotocin-induced T1D rats offered similar protective qualities, including improved renal hypertrophy, reduced polyuria and decreased urinary protein and vitamin D losses, in addition to significantly raising serum 25D concentrations. Future studies will investigate a combinatorial diet, containing whole egg and

resistant starch, to determine whether these two dietary interventions result in an additive effect with respect to renal protection and vitamin D balance.

Interestingly, whole egg consumption was without effect on urinary parameters in the T2D studies discussed in this dissertation; however, maintenance of circulating 25D concentrations in ZDF rats was still achieved. Importantly, we demonstrated that vitamin D derived from whole egg may be more effective than an equivalent amount of supplemental cholecalciferol added to a casein-based diet at maintaining serum 25D concentrations. Furthermore, in the T2D studies discussed, ZDF rats fed a whole egg-based diet exhibited a significant reduction in cumulative body weight gain compared to ZDF rats fed a casein-based diet. Individuals with T2D are at an increased risk for cardiovascular disease (CVD). Additionally, there is a high prevalence of overweight and obesity among the T2D population. Treatment for T2D focuses on lifestyle and dietary modifications as a means to control blood glucose concentrations, reduce body weight and minimize CVD risk (7). The results discussed in this dissertation suggest that inclusion of whole egg in the diet of individuals with T2D may support efforts in attaining healthy weight goals.

Factors accounting for vitamin D deficiency in IBD include insufficient dietary intake, inflammation, diarrhea and malabsorption; thus, malnutrition is common in individuals with IBD (8). Dietary recommendations for individuals with IBD are personalized and focus on restoring nutritional status and alleviating symptoms (9). The findings of our studies demonstrate that inclusion of dried whole egg, in the diet of rats with and without DSS-induced colitis, was more effective than an equivalent amount of supplemental cholecalciferol at increasing serum 25D concentrations. Therefore, egg

consumption may be an optimal source of nutrients, specifically vitamin D, to support overall health in individuals with IBD.

Considering the worldwide trends in vitamin D deficiency, the findings from these studies demonstrate the ability of a dietary intervention to successfully and significantly impact circulating 25D concentrations of individuals with chronic diseases characterized by vitamin D deficiency. More so, improvements in vitamin D status have the potential to mitigate disease complications. A dose response study is currently underway to identify the specific quantity of egg consumption that is efficacious with respect to influencing vitamin D status and may support new dietary recommendations targeting vitamin D deficiency prevention.

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APPENDIX LIST OF ABBREVIATIONS

1,25D	1,25-dihydroxycholecalciferol
25D	25-dihydroxycholecalciferol
APC	Antigen presenting cells
AMP	Antimicrobial peptides
BMI	Body mass index
CAS	Casein-based diet
CAS+D	Casein-based diet supplemented with cholecalciferol
CD	Crohn's Disease
CGM	Continuous glucose monitor
CHD	Coronary heart disease
CVD	Cardiovascular disease
CYP2R1	Cytochrome p450, family 2, subfamily R, member 1
CYP27A1	Cytochrome p450, family 27, subfamily A, member 1
CYP27B1	Cytochrome p450, family 27, subfamily B, member 1
Dab2	Disabled-2
DBP	Vitamin D-binding protein
DFE	dietary folate equivalent
DPP	Diabetes prevention program
DRIP	D-receptor interacting proteins
DSS	Dextran sulfate sodium
EIM	Extraintestinal manifestations
ER	Endoplasmic reticulum

FoxP3+	Forkhead box protein P3 positive
FMT	Fecal microbial transplant
GI	Gastrointestinal
GFR	Glomerular filtration rate
GLUT2	Glucose transporter-2
GLUT4	Glucose transporter-4
HbA1c	Hemoglobin A1c
HOMA-IR	Homeostatic model assessment - insulin resistance
HLA	Human leukocyte antigen
HMG-CoA	3-hydroxy-3-methylglutaryl CoA
HPA	Hypothalamus-pituitary-adrenal
IBD	Inflammatory bowel disease
IBD-AID	Inflammatory bowel disease anti-inflammatory diet
IEC	Intestinal epithelial cells
IgA	Immunoglobulin-A
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-10	Interleukin-10
IOM	Institutes of Medicine
IRS	Insulin receptor substrate
miRNA	MicroRNA
NFAT5	Nuclear factor of activated T-cells 5
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells

NOD	Non-obese diabetic
NOD2	Nucleotide-binding oligomerization domain containing 2
PTH	Parathyroid hormone
RAE	Retinol activity equivalents
RCT	Randomized control trials
RDA	Recommended dietary allowance
RXR	Retinoid x receptor
SREBP-2	Sterol regulatory element-binding protein-2
STZ	Streptozotocin
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TLR	Toll-like receptors
TMAO	Trimethylamine N-oxide
TNF- α	Tumor necrosis factor-alpha
TJ	Tight junction
UC	Ulcerative colitis
UVB	Ultraviolet B
VDR	Vitamin D receptor
VDRE	Vitamin D responsive element
VITAL	The vitamin D and omega-3 trial
WE	Whole egg-based diet
ZDF	Zucker diabetic fatty