RESEARCH ARTICLE

Effect of supplementation with vitamin D_3 on glucose production pathways in human subjects

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Scope: Research reports suggest that vitamin D affects glucose and insulin metabolism; however, the exact mechanisms are unclear. ^{2}H NMR analysis of monoacetone glucose (MAG) after tracer administration provides a non-invasive method of profiling hepatic glucose metabolism. This study examined the effects of supplementation with vitamin D_3 on contribution of glycogenolysis to glucose production.

Methods and results: Tracer administration and biofluid collections were performed with eight healthy females before and following a 4-wk vitamin D_3 administration period. Following an overnight fast subjects ingested deuterated water and acetaminophen. Full void urine samples were collected after 4 h. ^{2}H NMR spectra of urinary monoacetone glucose were acquired to determine the contribution of glycogenolysis to glucose production. The mean contribution of glycogenolysis to glucose production was $60 \pm 13\%$. Supplementation with vitamin D_3 had no effect on hepatic glucose production. Regression analysis revealed a significant relationship between carbohydrate intake and the contribution of glycogenolysis ($\beta = 0.914$, $p = 0.004$).

Conclusion: In conclusion, we saw no changes in the percentage contribution of glycogenolysis following supplementation with vitamin D_3. The reproducibility of our results and the non-invasive nature of the method highlight the potential for this method in assessing mechanistic modes of action in future nutritional interventions.

Keywords:
^{2}H NMR / Gluconeogenesis / Glycogenolysis / Urine / Vitamin D

1 Introduction

Nutritional systems biology describes the analysis and integration of all nutritional processes and is regarded as one of the major goals of modern nutrition research [1]. A key aspect to the development of models of nutritional responses in nutritional systems biology is the accurate quantification of metabolic fluxes. This can be partly addressed by challenge tests, such as the oral glucose tolerance test, which measures time-dependent changes in plasma metabolite and insulin concentrations following the ingestion of a glucose load. However, while providing an overview of systemic glucose metabolism this approach fails to provide direct information about hepatic glucose metabolism. In the post-absorptive state, glucose appearance is dependent on endogenous glucose production, which is sustained by a combination of hepatic glycogenolysis and gluconeogenesis. For this reason, the comprehensive assessment of glucose metabolism should include measurements of the metabolic pathways of hepatic glucose production. In humans, fluxes through pathways are typically evaluated by administration of precursors enriched with ^{2}H or ^{13}C in conjunction with MS or NMR analysis. This type of flux-based analysis provides valuable information to complement measurements of plasma glucose clearance and is beginning to enhance the understanding of the pathogenesis of diseases such as type 2 diabetes [2, 3].

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Abbreviations: 25(OH)D, 25 hydroxyvitamin D; **AG,** acetaminophen glucuronide; **^2H_2O,** deuterated water; **G6P,** glucose 6-phosphate; **MAG,** monoacetone glucose; **TAG,** triglyceride

Deuterated water (^2H_2O) is a stable isotope tracer, which when administered orally provides a direct measure of the contributions of glycogenolysis and gluconeogenesis to endogenous glucose production (see Fig. 1) [4, 5]. Typically, metabolic information is derived from the analysis of the ^2H-enrichment distribution of plasma glucose. More recently, a method has been developed to use urine samples for calculation of glucose fluxes (Fig. 1) [6, 7]. This method is based on oral administration of ^2H_2O and acetaminophen. In the liver, acetaminophen is metabolized to acetaminophen glucuronide (AG) and collection in the urine can be used for estimation of the isotopic labeling pattern of intrahepatic glucose. The non-invasive nature of this technique makes it an attractive approach for use in nutritional interventions.

There is accumulating evidence to suggest that low vitamin D status, reflected by circulating 25 hydroxyvitamin D [25(OH)D] concentrations less than 50 nmol/L [8–10], may play a role in the development of insulin resistance and type 2 diabetes [11–14]. Cross-sectional studies have reported inverse associations between 25(OH)D concentrations and several markers of glucose and insulin metabolism including fasting glucose and insulin concentrations, as well as with measurements of glucose and insulin during an oral glucose tolerance test and HOMA score (the homeostatic model assessment of insulin resistance) [15–17]. Furthermore, prospective research showed that baseline 25(OH)D concentrations were inversely associated with future risk of impaired fasting glucose and insulin resistance [16]. A number of interventions with vitamin D have been performed and, although results are inconsistent, changes in insulin concentrations and markers of insulin sensitivity have been described [18, 19]. Proposed mechanisms by which vitamin D (as the active metabolite, $1,25(OH)_2D$) exerts its effects include alterations on β-cell function, insulin sensitivity and glucose metabolism. While the majority of the literature focuses on changes in relation to insulin, decreased fasting glucose concentrations have also been reported following vitamin D treatment [20, 21]. To our knowledge no studies to date have examined mechanisms that may link vitamin D status and fasting glucose production. To this end, the present study was designed to examine the effects of vitamin D supplementation on hepatic glucose metabolism in young adult females. Using the non-invasive methods described above, the sources contributing to fasting glucose production were examined before and after a 4-wk vitamin D_3 administration period.

2 Materials and methods

2.1 Materials

Deuterium oxide (^2H_2O, 70%) was purchased from Cambridge Isotopes (Andover, MA, USA). Acetaminophen (1000 mg tablets) was purchased from a local pharmacy. DSC-18 SPE gel and all other chemicals were obtained from Sigma-Aldrich Ireland (Wicklow, Ireland).

2.2 Study design

Eight healthy females were recruited at University College Dublin. The protocol was approved by the Human Research

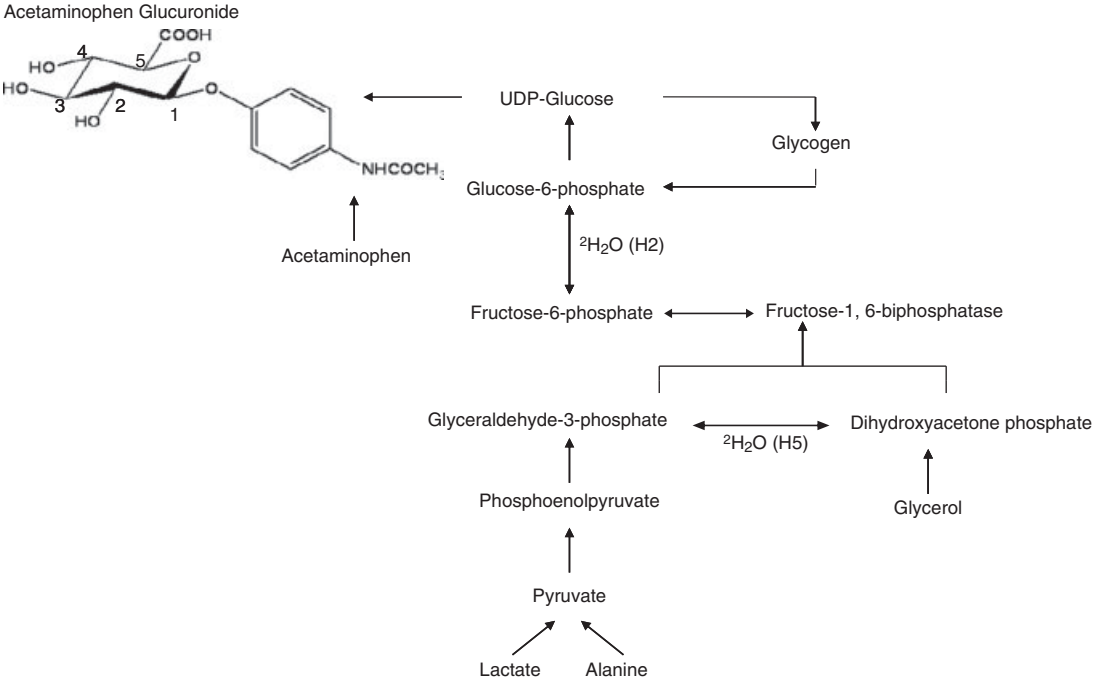

Figure 1. Metabolic pathways involved in fasting hepatic glucose metabolism and the formation of AG after deuterium (^2H_2O) and acetaminophen ingestion.

Ethics Committee in University College Dublin (HREC-39-06). All volunteers attended a screening session at which their height and weight were measured and a fingerpick blood sample was taken to measure haemoglobin. Exclusion criteria included a BMI <18.5 or >30.0 (kg/m^2), iron deficiency anaemia (hemoglobin <11 g/dL for females), any chronic or infectious disease and any prescribed medication for such, pregnant or lactating females, and persons using hormone replacement therapy. Contraceptive pills were permitted. Upon successful completion of screening, participants provided written informed consent. Tracer administration and biofluid collections were performed on two occasions, prior to and following 4 wk of supplementation (April 2008) with 15 μg of vitamin D_3 daily (which is the current recommended daily allowance for adults; Institute of Medicine, 2010). The vitamin D_3 and matching placebo, produced by Banner Pharmacaps (Tilburg, The Netherlands) were food grade and consumed in capsule form and were identical in appearance and taste. The vitamin D_3 content of the capsules was independently confirmed by laboratory analysis (Consultus, Glanmire, Cork, Ireland). Dietary intake was assessed by the use of 3-day estimated food record. Food diaries were coded and data was entered into the Weighed Intake Software Program (Tinuviel Software, Anglesey, UK) for analysis. Weighed Intake Software Program uses data from the McCance & Widdowson's "The Composition of Foods" plus supplemental volumes to generate nutrient intake data [22].

2.3 Tracer and acetaminophen administration and biofluid collection

Following an overnight fast, subjects reported to the research center at 8 a.m. Volunteers emptied their bladders before commencement of protocol. Over the first hour (8–9 a.m.), the tracers were given orally in three divided doses. All subjects received a total of 5 g/kg body water ^2H_2O. With the first dose of tracers, the patients also received 1000 mg of acetaminophen in the form of two paracetamol tablets. At 10 a.m., 11 a.m. and 12 p.m. 12 mL of blood was collected in tubes containing lithium heparin (Becton Dickinson, Oxford, UK). All samples were inverted eight times, placed directly on ice and processed within 1 h. Samples were centrifuged at $1800 \times g$ for 10 min at 4°C and aliquoted. A full void urine sample was collected at 12 p.m., and the volume recorded. Samples were stored at −80°C until subsequent analysis.

2.4 Sample preparation for NMR analysis

AG from urine samples was derivatized to acetaminophen-glucuronide methyl ester with some minor alterations to methods described previously [6]. An overview of the chemical reactions leading to the final end-product, monoacetone glucose (MAG) is shown in Fig. 2.

2.5 AG isolation

The full void urine sample (90.30 ± 48.49 mL) was defrosted and immediately sterilized by treatment with acetone for 2 h. The pH of the urine was then adjusted to pH 4.5 with 60% perchloric acid and the volume was reduced by 50% using rotary evaporation at 50°C and 30 mmHg. This solution was diluted to its original volume with water and treated with 10 000 U of urease. The pH of the sample was maintained near 7 by adding drops of 60% perchloric acid every 30–60 min until the reaction was complete and the pH

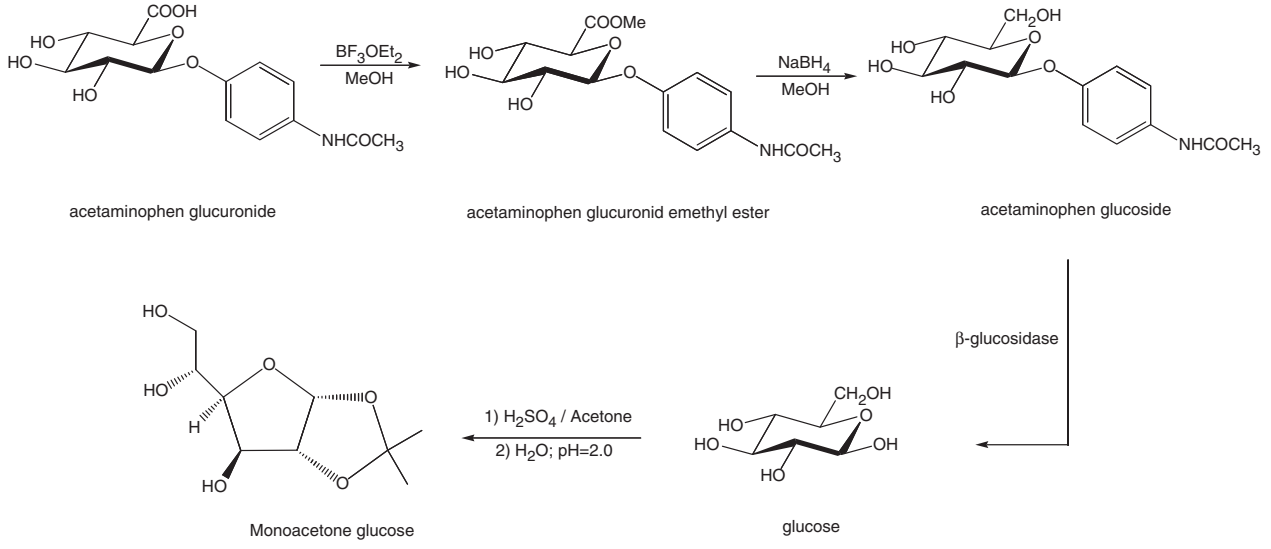

Figure 2. Reaction scheme for the conversion of AG to MAG.

stabilized. Finally, the pH was reduced to 4.5, the solution was filtered, lyophilized to dryness and refrigerated until further use.

Acidic water (pH 2.5) was prepared by adding 1 mL of concentrated phosphoric acid to 700 mL of deionized water. Thirty grams of SPE gel was loaded into a glass chromatography column and treated with 50 mL of 100% methanol followed by 100 mL of acidic water. The freeze-dried, urea-free urine sample was dissolved in a minimum amount of water (approximately 10–13 mL) and the pH was adjusted to 2–2.5 with 60% perchloric acid. The sample was loaded onto the column and washed with 120 mL of acidic water, 120 mL of 10% methanol in acidic water, and finally 120 mL of 100% methanol. The 10% methanol washing was collected, the pH was adjusted to 7 and the solvent was removed under vacuum to give a light-colored solid. This represented 70–88% of total urinary glucuronide as measured by HPLC.

2.6 Conversion of AG to MAG

The purified AG was dissolved in 50 mL of dry methanol, 300 μL of BF_3OEt_2 (neat) was added drop-wise to the reaction mixture. The reaction was stirred at room temperature and monitored by TLC (85:15 ethyl acetate: methanol; r.f. of the ester product 0.45) until total conversion to the ester was obtained (approximately 24 h).

Following conversion to the ester, $NaBH_4$ was added (in 60 mg portions every 10 min) to the same reaction flask until the reduction to alcohol was completed. Again, TLC was used to monitor the reaction and determine its endpoint. The pH of the solution was adjusted to 2 with concentrated HCl and the solvent was removed under vacuum to give a brown residue. The residue was washed three times with 2 mL of methanol and any remaining salt was removed by filtration. The washes are combined and evaporated.

The glucoside was dissolved in 25 mL of water, and the pH was adjusted to 7.4 and stirred overnight with 10 000 U of β-glucosidase. This solution was lyophilized and re-dissolved in a minimal amount of water and applied to a 5 g SPE column, prepared as described above. The glucose was eluted with 40 mL of water leaving coloured impurities behind. The colourless wash was lyophilized to a white solid.

The glucose/salt solid was suspended in 10 mL of dry acetone and 0.5 mL of concentrated H_2SO_4 was added. The reaction mixture was stirred for 8 h at room temperature. Ten milliliters of water were added to the reaction and the pH adjusted to 2.0 with 5 M KOH. The solution was stirred for another 48 h, the pH was adjusted to 8.0 with 5 M KOH and the solvent was removed under vacuum. The resulting solid was washed five times with 3 mL of boiling ethyl acetate. The washings were combined and the solvent was removed under vacuum. The residue was dissolved in 1 mL of a mixture of water/methanol in a ratio of 10:1 and applied to a 5 g SPE column prepared by washing with 20 mL of methanol followed by 60 mL of water. The column was washed with 20 mL of water and 40 mL of 10% methanol to remove pure MAG. The 10% methanol fraction was collected and lyophilized to dryness.

2.7 ^{2}H NMR spectroscopy

The samples were dissolved in 600 μL of ACN and 50 μL of deuterium-depleted water. Spectra were acquired on a 600 MHz Varian NMR spectrometer operating at 25°C. Proton-decoupled ^{2}H NMR spectra were acquired without field-frequency lock in blocks of 256 scans for a total of 160 blocks. The spectra were combined and fourier transformed using VnmrJ (NMR processing software, version3, Varian, Oxford, UK) software.

2.8 Analysis of ^{2}H spectra

Integration of the NMR spectra was performed manually using vnmrj software. The glucuronide moiety of acetaminophen is assumed to be derived from a hepatic uridine diphosphate-glucose pool that is in isotopic steady state with hepatic glucose 6-phosphate (G6P). G6P derived from glycogenolysis has deuterium in position 2 only, as a result of exchange between G6P and fructose-6-phosphate, whereas, G6P derived from gluconeogenic precursors, have deuterium incorporated in both positions 2 and 5 [7]. Hence, the ratio of the integrated area of ^{2}H enrichment in positions 5 and 2 (H5/H2) of the^{2}H NMR spectra of MAG reflects the fractional contribution of gluconeogenesis to fasting glucose production. The proportions of glucose derived from glycogenolysis and gluconeogenesis pathways are given by the following equations:

$$\text{Gluconeogenesis fraction}: \ H5/H2 \quad (1)$$

$$\text{Glycogenolysis fraction}: \ 1-(H5/H2) \quad (2)$$

2.9 Biochemical analyses of blood samples

Blood collected at 12 p.m. was analyzed for plasma glucose, total cholesterol, HDL cholesterol and triglyceride (TAG) using an automated clinical analyzer (Randox RX Daytona Clinical Analyser, Crumlin, Antrim). Plasma concentrations of 25(OH)D were determined independently using an ELISA (OCTEIA 25-Hydroxy Vitamin D; Immuno Diagnostic Systems, Boldon, UK). The quality and accuracy of serum 25(OH)D analysis in the UCC laboratory is assured on an ongoing basis by participation in the Vitamin D External Quality Assessment Scheme (DEQAS, Charing Cross Hospital, London, UK).

2.10 Statistical analysis

Data are presented as mean ± SD. Statistical analysis was performed using SPSS® version 15.0 for Windows (SPSS®, Chicago, IL, USA). Paired samples *t*-tests were used to examine the effects of vitamin D supplementation on the fractional contribution of glycogenolysis and gluconeogenesis to endogenous glucose production and the biochemical markers measured in plasma samples. $p < 0.05$ was considered to be statistically significant. Linear regression analysis was performed to examine relationships between glycogenolysis, plasma biomarkers and dietary intake.

3 Results

3.1 Subject characteristics

Eight females were recruited to the study with an average age of 21 ± 2 years and BMI of 21.64. ± 1.43 kg/m^2 (Table 1). Following the 4-wk intervention with vitamin D_3 (15 μg/day) there was a significant increase in plasma 25(OH)D levels from 55.1 ± 29.8 to 70.4 ± 22.3 nmol/L.

3.2 Conversion of AG to MAG

The total yield of glucose from AG typically varied between 50 and 80% (see Fig. 2). The yield of MAG varied between 40 and 80% depending on the sample. For two samples from the same subject, derivatization of glucose to MAG was incomplete; as a result accurate ^{2}H NMR analysis could not be achieved, and the spectra were removed from further analysis.

3.3 The contribution of glycogenolysis and gluconeogenesis to glucose production

^{2}H NMR spectra of MAG derived from AG generated well-resolved ^{2}H MAG resonances. Overall, the fractional contribution of glycogen to glucose production was similar for both time points (pre- and post-Vitamin D administration); except for two subjects (numbers 3 and 5) whose percentage contribution from glycogenolysis varied substantially (Figs. 1 and 3). Statistical analysis revealed no significant difference in the percentage contribution of glycogenolysis to glucose production following the intervention period (Table 2). Overall, the mean fraction of glucose derived from glycogenolysis and gluconeogenesis was 60 ± 13% and 40 ± 13%, respectively.

Table 1. Subject characteristics[a)]

	Females ($n = 8$)
Height (m)	1.61 ± 0.18
Weight (kg)	60.65 ± 6.93
BMI (kg/m^2)	21.64 ± 1.43
Age (y)	21 ± 2

a) All values are mean ± SD.

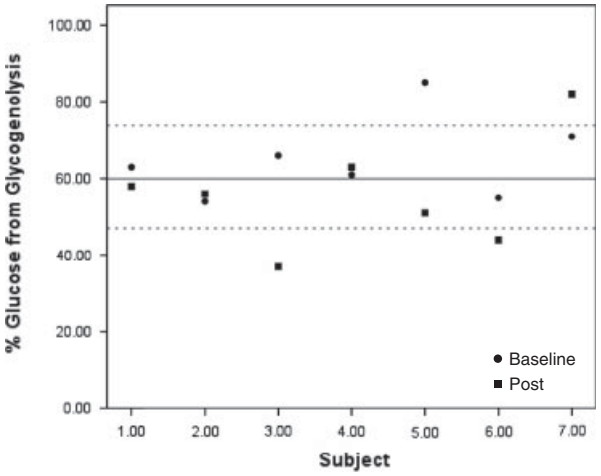

Figure 3. Percentage of glucose production from glycogenolysis for each subject studied at baseline and following a 4-wk vitamin D intervention study. The solid line corresponds to the mean contribution of glycogenolysis to glucose (60%), and the dashed lines represent the SD (13%).

Table 2. Estimates of the percentage contribution of glycogenolysis to glucose production and serum 25(OH)D for each subject at baseline and after a 4-wk vitamin D_3 (15 μg/day) intervention study[a),b)]

Subject	Glycogenolysis (%)		Serum 25(OH)D (nmol/L)	
	Baseline	Post	Baseline	Post
1	63	58	59.3	73.6
2	54	56	58.0	91.8
3	66	37	123.8	108.2
4	61	63	33.1	44.6
5	85	51	43.3	63.3
6	55	44	54.5	79.9
7	71	82	37.0	51.8
Mean ± SD	65 ± 11	56 ± 14	58.4 ± 30.6	73.3 ± 11.6*

a) There was no significant difference between baseline and post values for the percentage contribution of glycogenolysis to glucose production ($p = 0.199$).

b) *A paired sample *t*-test indicated a significant difference between baseline and post serum 25(OH)D nmol/L concentrations ($p = 0.010$, $n = 7$). Mean values for 25(OH)D are different to those in Table 3 as only $n = 7$ subjects were used here due to the incomplete conversion of glucose to MAG.

Table 3. Plasma biomarkers at baseline and following a 4-wk vitamin D_3 (15 μg/day) intervention study[a)]

	Baseline	Post	*p*-Value
25(OH)D (nmol/L)	55.1 ± 29.8	70.4 ± 22.3	0.020
Glucose (mmol/L)	4.95 ± 0.29	4.97 ± 0.32	0.927
Total cholesterol (mmol/L)	3.83 ± 0.75	3.77 ± 0.68	0.802
HDL cholesterol (mmol/L)	1.67 ± 0.41	1.63 ± 0.32	0.636
TAG (mmol/L)	0.60 ± 0.27	0.64 ± 0.43	0.694

a) All values are mean ± SD ($n = 8$), *p* values are based on paired sample *t* test.

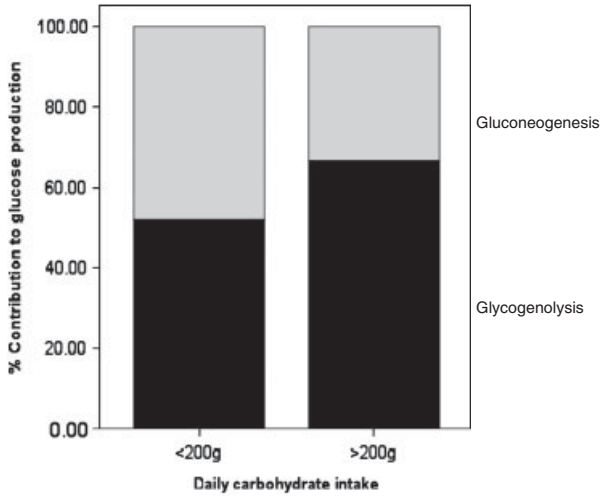

Figure 4. Mean contribution from glycogenolysis and gluconeogenesis to fasting glucose production for individuals consuming less than 200 g of carbohydrate per day and individuals consuming more than 200 g of carbohydrate per day. ANOVA indicated a significant difference in mean contribution from glycogenolysis between groups ($p = 0.022$).

3.4 The relationship between glycogenolysis, plasma biomarkers and dietary intake

Following the 4-wk intervention with 15 μg/day of vitamin D_3 there was a significant increase in 25(OH)D concentrations (Table 3). However, vitamin D supplementation did not significantly alter glucose, total cholesterol, HDL cholesterol or TAG concentrations (Table 3). There was no significant relationship between fasting plasma glucose and the percentage contribution of glycogenolysis to glucose production. Similarly, none of the plasma biomarkers measured was significantly associated with the percentage contribution of glycogenolysis to glucose production.

Regression analysis revealed a significant relationship between carbohydrate intake (g) and percentage contribution of glycogenolysis to glucose production ($\beta = 0.914$, $p = 0.004$). Comparing subjects with high and low carbohydrate intakes confirmed these findings. Individuals consuming greater than 200 g of carbohydrate per day had a significantly higher percentage contribution from glycogenolysis (67 ± 7%) compared to subjects consuming less than 200 g of carbohydrate per day (52 ± 3%) ($p = 0.022$, Fig. 4). Interestingly, a similar relationship was not evident between fasting glucose concentrations and carbohydrate intake.

4 Discussion

The ^2H_2O method is currently the most widely accepted method for estimating glycogenolysis and gluconeogenesis in humans [5–7, 23, 24]. A non-invasive method using urinary AG to monitor enrichment of hepatic glucose from ^2H_2O has been developed and was employed with minor modifications in the current study to investigate changes following a nutritional intervention [6]. Results from analysis of urinary glucuronide suggest that the mean contribution of glycogenolysis to glucose production for healthy young females after an overnight fast is 60 ± 13%. Our findings are in agreement with data from previous studies where the reported fraction of glucose derived from glycogenolysis is 51 ± 11% [6].

The extent of normal human variation and the influence of variation on metabolic processes have been extensively reported in the literature. Indeed, results from the present study and others indicate that variation exists in the fractional contribution of glycogenolysis to fasting glucose production. There are several potential contributory factors, ranging from inter-subject genetic variation to time-dependent changes in physiology, which could arise both within and between individuals. Different durations of fasting will have an impact on the contribution of glycogenolysis [25]. In the current study, subjects were asked to begin fasting at the same time. However, the actual time since their last meal could be different, which may account for part of the variation in estimates of gluconeogenesis observed in the present study. Variation in the contribution of glycogenolysis to glucose production could also be a reflection of dietary composition. Data from the present study indicates that the contribution from glycogenolysis increases with increasing carbohydrate intake. A higher carbohydrate intake will result in increased glycogen stores, hence a higher contribution of glycogenolysis to fasting glucose production. These findings agree with a previous study

designed to investigate the effect of dietary carbohydrate content on fasting glucose metabolism [26]. In this randomized cross-over study six healthy males consumed three isocaloric diets with 85, 44 and 2% of energy from carbohydrate. Contributions to glycogenolysis and gluconeogenesis were measured in plasma glucose revealing that the absolute contribution from glycogenolysis was $57 \pm 3\%$ for the high carbohydrate diet, $51 \pm 2\%$ for the control diet and $34 \pm 4\%$ for the low carbohydrate diet. More recently, Burgess and colleagues reported a similar effect when comparing two energy-restricted diets [27]. Carbohydrate restriction increased the contribution from gluconeogenesis and reduced the contribution from glycogenolysis. The novelty with the current data is that the relationship between dietary intake and glycogenolysis was significant with estimates of habitual carbohydrate intake.

The next step in the present research was to determine the effects of the vitamin D_3 intervention on the contribution of glycogenolysis to glucose production. Epidemiological studies have shown relationships between low vitamin D status and markers of disturbed glucose metabolism [15–17]. Furthermore, evidence from vitamin D intervention studies have shown changes in fasting glucose concentrations following supplementation. In one study, intravenous administration of vitamin D to pregnant women with gestational diabetes decreased fasting glucose levels [20]. In addition, post hoc analysis of a recent long-term intervention in patients with osteoporosis showed that individuals with impaired fasting glucose at baseline demonstrated a smaller increase in fasting glucose and insulin resistance when treated with vitamin D plus calcium rather than placebo [28]. In contrast the present study showed that increasing serum 25(OH)D concentrations had no significant effect on fasting glucose concentrations in healthy volunteers, consistent with other research findings [29]. Furthermore, there was no significant difference in the fractional contribution of glycogenolysis and gluconeogenesis to glucose production following vitamin D supplementation. To our knowledge, this is the first study of its kind to examine the effects of vitamin D on pathways of hepatic glucose metabolism using the ^2H_2O method.

Mechanistic evidence from previous research suggests that vitamin D (as the active form, $1,25(OH)_2D$) may exert its effects on glucose metabolism through increased insulin secretion [30] and improved insulin sensitivity [31, 32]. Indeed, these findings are supported by results from other human intervention studies that show improvements in markers of insulin resistance with vitamin D supplementation [18, 19]. While the present study failed to highlight changes in fasting glucose concentrations or changes in the contribution of different sources to fasting glucose production, insulin sensitivity per se was not examined. In addition, drawing conclusions from individual supplementation studies is confounded by several factors including the metabolic characteristics of subjects (e.g. diseased or healthy), the dose of vitamin D and the length of the supplementation period. The fact that subjects included in the present study were all healthy with a mean serum 25(OH)D concentration >50 nmol/L prior to the intervention may account for the lack of effect of vitamin D supplementation. The possibility remains that vitamin D supplementation in vitamin D-deficient individuals or in those at risk of diseases related to glucose metabolism could result in significantly different findings and future work will address this. Additionally, it is possible that phenotyping individuals prior to inclusion in tracer-based studies may help identify a type of individual that has positive changes in hepatic glucose production pathways following supplementation with vitamin D. Indeed, recently we demonstrated that a phenotyping approach could be used to identify a vitamin D-responsive ''metabotype'' [33].

In conclusion, we demonstrated a practical, reproducible, non-invasive method for quantifying the sources of hepatic glucose production that can be readily adapted for inclusion in human nutritional intervention studies. Vitamin D supplementation had no effect on the contributions of glycogenolysis and gluconeogenesis to fasting glucose production. Overall, ^2H_2O and acetaminophen administration offers a viable method for assessing the biological implications of interventions with respect to hepatic glucose production pathways, which will undoubtedly play a role in the future of nutritional systems biology.

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The authors have declared no conflict of interest.

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