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Vitamin D and Vitamin K Concentrations in Human Brain Tissue Are Influenced by Freezer Storage Time: The Memory and Aging Project

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ABSTRACT

Background: Vitamins D and K, which are present in human brain, may have a role in neurodegenerative disease.

Objectives: Given the interest in measuring nutrient concentrations in archived brain samples, it is important to evaluate whether freezer storage time affects these concentrations. Therefore, we evaluated differences in vitamin D and vitamin K concentrations in human brain samples stored for various lengths of time.

Methods: Postmortem brain samples were obtained from 499 participants in the Rush Memory and Aging Project (mean age 92 y, 72% female). Concentrations of vitamins D and K and their metabolites were measured in 4 regions (midtemporal cortex, midfrontal cortex, cerebellum, anterior watershed white matter) using LC-MS/MS and HPLC, respectively. The predominant forms were 25-hydroxycholecalciferol [25(OH)D_3] and menaquinone-4 (MK4). ANOVA was used to determine if concentrations differed according to storage time.

Results: The geometric mean of the mean 25(OH)D_3 concentration (across 4 regions) in brains stored for 1.1 to 6.0 y did not differ from that in brains stored ≤1.0 y (all $P \geq 0.37$), whereas 25(OH)D_3 in brains stored >6.0 y was 31–40% lower ($P \leq 0.003$). MK4 had similar results, with the geometric mean MK4 concentration in the brains stored ≥9.0 y being 48–52% lower than those in brains stored ≤1.0 y ($P \leq 0.012$). The 25(OH)D_3 and MK4 concentrations were positively correlated across all 4 regions (all Spearman $\rho \geq 0.79$, $P < 0.001$).

Conclusions: 25(OH)D_3 and MK4 appear to be stable in brain tissue from older adults stored at -80°C for up to 6 and 9 y, respectively, but not longer. Freezer storage time should be considered in the design and interpretation of studies using archived brain tissue. *J Nutr* 2021;151:104–108.

Keywords: Alzheimer disease and related dementias, vitamin D, vitamin K, storage, stability, brain

Introduction

Vitamin D and vitamin K are fat-soluble nutrients that have been implicated in Alzheimer disease and related dementias (ADRD). Vitamin D and the vitamin D receptor (VDR) are present in human brain tissue (1–3). In rodent experiments, 1,25-dihydroxyvitamin D_3 upregulated neurotrophic factors (4), prevented excitotoxicity, and exerted anti-inflammatory activities in brain tissue (5). Vitamin K, which is also present in human brain tissue, predominantly in the form of menaquinone-4 (MK4) (6, 7), functions as an enzymatic cofactor for vitamin K–dependent proteins in cerebral tissue. In rodents, multiple vitamin K–dependent proteins are present in the cerebral cortex and other brain regions (8). Vitamin K is also involved in sphingolipid metabolism (9). Sphingolipids are important membrane constituents that have a role in cognitive behavior and are abundant in brain tissue. These mechanisms are supported by population-based studies that found vitamin D and vitamin K insufficiencies to be associated with an increased risk for AD/cognitive decline (10–15). It is therefore plausible for vitamin D and/or vitamin K to have a protective role in ADRD and cognitive decline. However, evidence would

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Abbreviations used: 1,25(OH)2D3, 1,25-dihydroxycholecalciferol; 25(OH)D3, 25-hydroxycholecalciferol; ADRD, Alzheimer disease and related dementias; AWS: anterior watershed white matter; CR, cerebellum; D_3, cholecalciferol; LOD, lower limit of detection; MAP, Memory and Aging Project; MF, midfrontal cortex; MK4, menaquinone-4; MT, mid-temporal cortex; RSD, relative standard deviation; VDR, vitamin D receptor.

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TABLE 1 Vitamin D and vitamin K concentrations in 4 brain regions of older adults in the MAP study[1]

	AWS	CR	MT	MF	Mean	% ND[2]
$25(OH)D_3$, pmol/g	0.9 (ND-4.9)	1.1 (ND-7.1)	1.1 (ND-6.4)	1.1 (ND-11.4)	1.1 (ND-5.5)	0
$1,25(OH)_2D_3$, pmol/g	ND (ND-0.8)	ND (ND-0.5)	ND (ND-0.3)	ND (ND-0.3)	ND (ND-0.3)	58
D_3, pmol/g	0.1 (ND-69.6)	0.2 (ND-18.7)	0.1 (ND-21.0)	0.2 (ND-20.9)	0.2 (ND-24.6)	22
MK4, pmol/g	0.6 (ND-42.8)	1.4 (ND-78.3)	1.3 (ND-43.6)	1.3 (ND-52.8)	1.2 (ND-47.8)	3
Phylloquinone, pmol/g	ND (ND-4.8)	ND (ND-1.8)	ND (ND-2.1)	ND (ND-1.7)	ND (ND-2.3)	83

[1] Data are the median (range) of the mean concentrations of the 4 regions, $n = 499$ subjects. The LODs are as follows: $25(OH)D_3$: 0.1 pmol/g; $1,25(OH)_2D_3$: 0.06 pmol/g; D_3: 0.06 pmol/g; MK4: 0.1 pmol/g; and phylloquinone: 0.1 pmol/g. $1,25(OH)_2D_3$, 1,25-dihydroxycholecalciferol; $25(OH)D_3$, 25-hydroxycholecalciferol; AWS, anterior watershed white matter; CR, cerebellum; D_3, cholecalciferol; LOD, lower limit of detection; MAP, Memory and Aging Project; MF, midfrontal cortex; MK4, menaquinone-4; MT, midtemporal cortex.

[2] Percentage of participants with nondetectable (below the assay LOD) concentrations.

be strengthened by a broader evaluation of these vitamin metabolites in human brain tissue.

The establishment of biospecimen repositories that include storage of human brain tissue has facilitated translational research in ADRD (16). As interest in conducting analysis of archived brain samples increases, it is important to evaluate how storage conditions might affect the analyses of interest in human brain tissue. The objective of this study was to evaluate the influence of freezer storage time on vitamin D and vitamin K concentrations in human brain tissue stored for up to 13 y.

Methods

Study population

Postmortem brain samples were obtained from 499 participants in the Rush Memory and Aging Project (MAP), a clinical-pathologic prospective epidemiologic cohort study of residents of retirement communities and public housing in the Chicago area (17). At enrollment, all MAP participants were free of clinically diagnosed dementia and agreed to risk factor assessment, annual clinical evaluation, and brain donation at death. Samples obtained for this study were from participants who died between 2004 and 2019. The median (range) storage time of the brain samples was 4.9 (0.4–13.0) y. At autopsy and brain dissection, the tissues were immediately frozen and not allowed to thaw during the dissection. The tissues were stored at –80°C until analysis. The MAP study was approved by the Institutional Review Board of Rush University Medical Center. This ancillary study was additionally approved by the Institutional Review Board at Tufts University Health Sciences.

Vitamin D and vitamin K analyses

Cholecalciferol (D_3) and its metabolites, $25(OH)D_3$ and 1,25-dihydroxycholecalciferol [$1,25(OH)_2D_3$], were measured using LC-MS/MS in 4 brain regions [midtemporal cortex (MT), midfrontal cortex (MF), cerebellum (CR), and anterior watershed white matter (AWS)] as previously described (2). The lower limits of detection (LOD) for this assay are as follows: vitamin D_3 0.06 pmol/g; $25(OH)D_3$ 0.1 pmol/g, and $1,25(OH)_2D_3$ 0.06 pmol/g (2).

Phylloquinone (vitamin K1) and MK4 (a metabolite of phylloquinone and a form of vitamin K2) were extracted and purified from the MT, MF, CR, and AWS using established methods (18). To improve separation, a C30 column was used as described previously (19). The assay LOD for both phylloquinone and MK4 is 0.1 pmol/g. The details of the sample preparation and detection are described in the **Supplemental Methods**.

With regard to quality control procedures, there are currently no universal vitamin D and vitamin K standards available to use as controls for analyzing these nutrients in human brain tissue. Therefore, we used in-house controls (human brain samples or serum with known amounts of each vitamin) with each batch of 20 samples to monitor assay drift. Calibration and internal standards were injected every 6–10 samples. The CV values for inter-assay precision of D_3, $25(OH)D_3$, and $1,25(OH)_2D_3$ were characterized by relative SDs of 10.2%, 9.3%, and 10.0%, respectively. Similarly, the interassay precision values of MK4 and PK were 10.4% and 9.6%, respectively. All samples were analyzed randomly, not in sequence order of year received.

Statistical analyses

Our main statistical analyses focused on $25(OH)D_3$ and MK4 because these were the primary forms of vitamin D and vitamin K detected in the human brain samples. All statistical analyses were conducted using SAS v 9.4. The mean $25(OH)D_3$ and MK4 concentrations in the 4 regions were calculated [henceforth referred to as mean $25(OH)D_3$ and mean MK4]. Prior to analysis, the distributions of mean $25(OH)D_3$ and mean MK4 were natural-log transformed to improve normality. General linear models (proc glm) were used to evaluate differences in mean $25(OH)D_3$ and mean MK4 concentrations according to storage time. Storage time was categorized into 1-y increments, with the samples stored ≤1.0 y serving as the reference group. A Bonferroni corrected P value of <0.005 was considered statistically significant (0.05/10 comparisons). Because population-based studies indicate an increase in vitamin D supplement use in the United States over the course of this study (20), we adjusted for vitamin D supplement use in a sensitivity analysis. Unfortunately, information on supplement use was not available for participants whose brains were stored for >8 y. To verify that the association between $25(OH)D_3$ and storage time was independent of vitamin D supplement use, vitamin D supplement use was included as a covariate in a sensitivity analysis. In a separate sensitivity analysis, we adjusted the models for age at death to verify that the results were not age dependent. The differences in $25(OH)D_3$ and MK4 across storage time categories were evaluated in the separate MF, MT, CR, and AWS regions in secondary analyses following the same approach. Spearman rank order correlation coefficients were used to evaluate the correlations of $25(OH)D_3$ and MK4, respectively, among the 4 regions. The $25(OH)D_3$ and MK4 concentrations were compared across the 4 regions in the brain samples stored ≤6 y and ≤9 y, respectively, using a repeated measures mixed-effect models (proc mixed).

Results

This study utilized brain tissue samples from 499 subjects with a median (range) age at death of 92 (63–108) y. The majority (72%) were female.

The primary form of vitamin D detected in human brain tissue was $25(OH)D_3$. Although detected, D_3 and $1,25(OH)_2D_3$ were below the assay LOD in 22% and 58% of participants, respectively (**Table 1**). The geometric mean of the mean $25(OH)D_3$ concentration in the 4 brain regions in brains stored for 1.1–6.0 y did not differ from the $25(OH)D_3$ in brains stored ≤6.0 y (all $P \geq 0.37$). However, the geometric mean of the mean $25(OH)D_3$ concentration in brains stored >6.0 y was significantly lower than that in the brains stored ≤1.0 y ($P \leq 0.003$) (**Figure 1**A). When vitamin D supplement

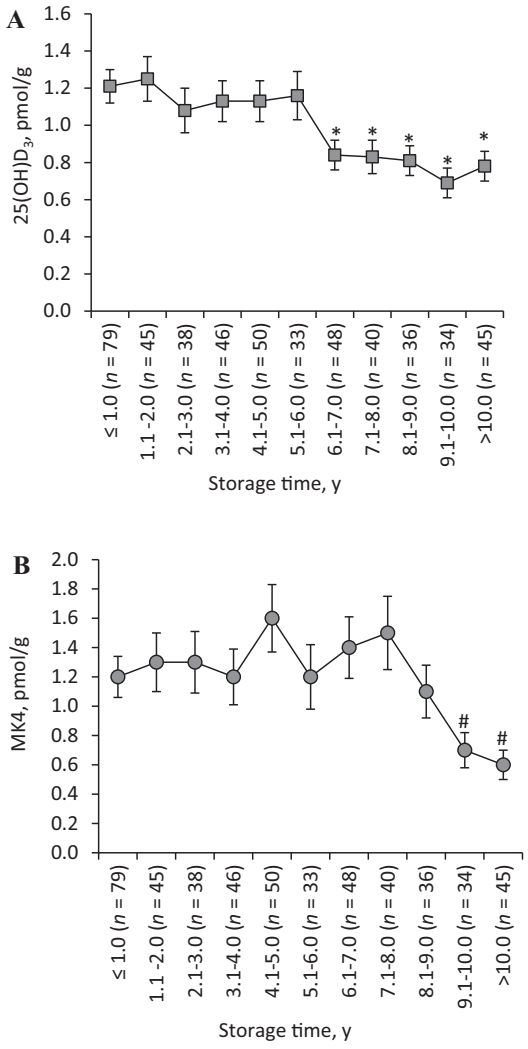

FIGURE 1 Brain 25(OH)D_3 (A) and MK4 (B) concentrations across 4 regions of brains stored at -80°C for various periods of time from older adults in the MAP study. Values are geometric mean of the 4 brain regions means ± SEM. Significantly different from reference group (≤1.0 y storage time): *$P \leq 0.003$; #$P \leq 0.001$. 25(OH)D_3, 25-hydroxycholecalciferol; MAP, Memory and Aging Project; MK4, menaquinone-4.

use was controlled for (using the 330 participants for whom information on supplement use was available), the association between mean 25(OH)D_3 and storage time did not appreciably change (**Supplemental Table 1**). The brains stored 6.1–8.0 y had significantly less 25(OH)D_3 than the brains stored ≤1.0 y.

TABLE 3 25(OH)D_3 and MK4 concentrations compared among 4 brain regions[1]

	25(OH)D_3, pmol/g store ≤6 y ($n = 292$)	MK4, pmol/g stored ≤9 y ($n = 415$)
AWS	1.0 (0.04)[a]	0.6 (0.04)[a]
CR	1.2 (0.04)[b]	1.3 (0.08)[b]
MT	1.2 (0.04)[b]	1.2 (0.08)[b]
MF	1.2 (0.04)[b]	1.2 (0.08)[b]

[1]Values are geometric mean (SEM). Labeled means in a column without a common letter differ, $P < 0.001$. 25(OH)D_3, 25-hydroxycholecalciferol; AWS, anterior watershed; CR, cerebellum; MF, midfrontal cortex; MK4, menaquinone-4; MT, midtemporal cortex.

MK4 was the primary form of vitamin K detected in human brain. Phylloquinone was below the LOD in >80% of participants (Table 1). The geometric mean of the mean MK4 concentration in the 4 brain regions in brains stored for 1.1–9.0 y did not differ from the MK4 in brains stored ≤1.0 y (all $P \geq 0.11$). However, the geometric mean MK4 concentration in the brains stored ≥9.0 y was significantly lower than the concentration in the brains stored ≤1.0 y ($P \leq 0.012$) (Figure 1B). Similar results were obtained when the associations of 25(OH)D_3 and MK4 with storage time were evaluated in the 4 regions separately (**Supplemental Tables 2 and 3**). Adjustment for age at death and sex did not change the results (data not shown).

The brain 25(OH)D_3 and MK4 concentrations were positively correlated across all 4 regions, respectively, with and without adjustment for storage time (all Spearman $\rho \geq 0.79$, $P < 0.001$) (**Table 2**). In the brains stored ≤6.0 y, the 25(OH)D_3 concentrations in the MT, MF, and CR regions were similar, whereas the 25(OH)D_3 in the AWS was significantly lower than in the other 3 regions ($P < 0.001$). In the brains stored ≤9.0 y, the MK4 concentrations in the MT, MF, and CR regions were similar, but the MK4 concentration in the AWS was significantly lower than concentrations in the other 3 regions (**Table 3**).

Discussion

The goal of this study was to evaluate the association of freezer-storage time with vitamin D and vitamin K concentrations, 2 nutrients mechanistically linked to ADRD and cognitive decline (2, 5, 6, 8, 9, 21) in human brain tissue. The concentrations of 25(OH)D_3, the primary form of vitamin D detected in human brain, were significantly lower in brains stored >6 y than in brains stored ≤1.0 y, suggesting that 25(OH)D_3 was stable in

TABLE 2 Spearman rank order correlations of 25(OH)D_3 and MK4 between brain regions of older adults in the MAP study[1]

	25(OH)D_3		MK4	
	Unadjusted	Adjusted for storage time	Unadjusted	Adjusted for storage time
AWS-CR	0.89	0.89	0.79	0.80
AWS-MF	0.91	0.90	0.83	0.82
AWS-MT	0.90	0.89	0.82	0.82
CR-MF	0.93	0.92	0.87	0.88
CF-MT	0.94	0.94	0.89	0.89
MF-MT	0.95	0.94	0.90	0.90

[1]All correlations are significant at $P < 0.001$. $n = 499$ subjects. 25(OH)D_3, 25-hydroxycholecalciferol; AWS, anterior watershed; CR, cerebellum; MAP, Memory and Aging Project; MF, midfrontal cortex; MK4, menaquinone-4; MT, mid-temporal cortex.

samples stored frozen at -80°C for up to 6 y. The concentrations of MK4, the primary form of vitamin K detected in human brain, were significantly lower in brains stored >9.0 y than in brains stored ≤1.0 y, suggesting MK4 was stable in samples stored frozen at -80°C for up to 9 y. These findings highlight the importance of considering freezer storage time in the design and interpretation of studies using stored brain tissue samples to link brain concentrations of these vitamin metabolites to neurodegenerative diseases.

To the best of our knowledge, this is the first report on the association of freezer storage time with vitamin D and vitamin K concentrations in human brain tissue. In contrast to our findings in brain, 25(OH)D_3 is reported to be stable in serum for up to 24 y stored frozen at −25°C and multiple freeze–thaw cycles (22–24). Unpublished data from our laboratory indicate that plasma phylloquinone is stable for ≥12 y in samples frozen at −80°C. The stability of nutrients in brain tissue may differ from stability in circulation because most nutrients are transported in serum/plasma bound to protein or lipoproteins. The brain is also a lipid-rich organ with barrier mechanisms required for normal functioning. Although the transport and storage of nutrients in brain tissue is not well understood, transport and metabolic considerations of nutrients could account for differences in the stability during freezer storage.

Our finding that MK4 was the predominant form of vitamin K in human brain is consistent with previous studies (6, 7). Although phylloquinone is the primary dietary and circulating form of vitamin K, its conversion to MK4 in brain tissue has been confirmed by use of rodent models and stable isotopes (25, 26). However, the human brain MK4 concentrations in our study were approximately 3–4 pmol/g lower than those reported by others (6, 7). This discrepancy may be related to study participant characteristics and/or analytical differences. We found that when a C18 column was used for separation of compounds of interest (18), there were coeluting peaks interfering with the MK4 peak on the chromatograms. When we modified the separation method to use a C30 column, the coeluting peaks that interfered with the MK4 peak were eliminated. The C30 column is designed for high-resolution separation of hydrophobic, long-chain compounds, such as MK4, and, based on our findings, is recommended for analysis of vitamin K forms in brain tissue. In a recently published study, serum phylloquinone was positively associated with cognitive function, but the MK4 brain concentration was not (6, 7). However, because the brain tissue samples used in this study were stored for >10 y before analysis, it is possible that the brain MK4 degraded during the storage time, which may have affected the results.

The 25(OH)D_3 and MK4 concentrations were highly correlated among the 4 brain regions analyzed, respectively. However, the concentrations of both vitamin forms were significantly lower in the AWS than in the MF, MT, and CR. The AWS region sampled contained all white matter, whereas the other regions sampled contained grey matter. Little is known about the distribution of these nutrients in white or grey matter or in different brain regions. The AWS may be particularly susceptible to vascular diseases and vascular risk factors (27), whereas the MF and MT cortexes are the neocortical regions most commonly affected by AD pathology (28, 29). Additional research is needed to clarify the differential roles, if any, of nutrients in different brain regions.

Important strengths of this study are the large sample size and state-of-the-art laboratory methodology used to quantify the vitamin D and K forms in 4 separate regions of the human brain. Because 25(OH)D_3 and MK4 were detected in nearly all of the brains analyzed, we were able to evaluate the effect of freezer storage time on these vitamin forms. A large number of participants had brain vitamin D_3, 1,25$(OH)_2D_3$, and phylloquinone concentrations below the assay LOD, which challenged our ability to evaluate how long-term storage affects these vitamin forms. However, the likelihood of having concentrations of vitamin D_3, 1,25$(OH)_2D_3$, and phylloquinone below the LOD did not appear to differ with storage time (data not shown). Information on vitamin D supplement use was not available for participants whose brains were stored for >8 y. However, our data suggest that vitamin D supplement use did not confound our main results. Information on vitamin K supplement use was not available on our participants. However, shifts in vitamin K supplement use would be less likely to affect the results because vitamin K supplement use has remained stable in the United States since 1999 (20). Circulating measures of vitamin D status and vitamin K status are also not currently available in MAP, which limits our understanding of how nutritional status of MAP participants is associated with the vitamin D and vitamin K brain concentrations.

In conclusion, the results of this study indicate concentrations of 25(OH)D_3 and MK4 in human brain samples from older adults stored at −80°C are affected by duration of storage time. The design and interpretation of any research focused on elucidating the roles of these nutrients in neurodegenerative disease using banked brain tissue samples need to consider how long the samples have been stored. Moreover, as the use of stored brain tissue in translational ADRD research increases (16), it will be important to evaluate the stability of other biochemical measures over long-term freezer storage to ensure scientific rigor.

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