7β-Hydroxycholesterol enhances the amyloidogenic pathway: implications for Alzheimer's disease

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ABSTRACT

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Purpose: Despite increasing evidence that oxysterols are elevated in Alzheimer's disease (AD), little is known about the effects of oxysterols on AD brain pathology. Further, the analysis of oxysterols has been challenged because of methodological limitations that allow sampling of only blood or CSF. Recently, hair cholesterol has been reported as a valid peripheral marker for cholesterol metabolism. Therefore, the aim of the present study was to investigate the relationship between hair 7β-hydroxycholesterol (7β-OHC) and AD in human and examine the effects of 7β-OHC treatment on the mouse brain.

Methods: 7β-OHC levels in the scalp hair of participants with normal cognition (NC, n = 82), mild cognitive impairment (MCI, n = 39), and AD (n = 81) were assessed using gas chromatography-mass spectrometry. The relationships between hair 7β-OHC levels and various clinical features representing cognitive status were analyzed. Upon stereotaxic injection of 7β-OHC into the hippocampus of mice, AD pathologies such as β-amyloid, tau, and neuroinflammation were analyzed. The behavioral effects of intracerebroventricular

injection of 7β-OHC were assessed using the touchscreen-based 5-choice serial reaction time task.

Results: Hair 7β-OHC levels were increased in AD patients than in NC individuals ($p = 0.001$). There were also significant correlations between hair 7β-OHC levels and Mini-Mental State Examination (MMSE) and Global Deterioration Scale (GDS) scores after controlling for age, sex, education, and statin use. The animal experiments showed that hippocampal 7β-OHC injection induced microglial and astrocyte activation and increased the levels of interleukin (IL)-1β and IL-6. Hippocampal 7β-OHC injection also promoted the amyloidogenic pathway of amyloid precursor protein (APP) processing by upregulating β-amyloid converting enzyme 1 (BACE1; β-secretase) while downregulating tumor necrosis factor alpha converting enzyme (TACE; α-secretase). Behavioral assessment showed that 7β-OHC treatment increased perseveration, which is compatible with the response observed in frontal lobe dysfunction.

Conclusion: The results of this study suggest that 7β-OHC levels in the scalp hair could serve as a convenient peripheral biomarker for AD. Given that the levels of accumulated steroid molecules in the hair reflect plasma levels during the past few months, this study also suggests that elevated plasma 7β-OHC could contribute to AD pathogenesis via increasing neuroinflammation and inducing the amyloidogenic pathway.

Key words: oxysterol, 7β-hydroxycholesterol, hair cholesterol, neuroinflammation, Alzheimer's disease

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I. INTRODUCTION

1. Alzheimer's disease (AD)

Alzheimer's disease (AD) is a debilitating neurodegenerative disease that results in a decline in memory and other cognitive functions. AD eventually results in an inability to cope with the demands of daily and social life. The disease is responsible for up to 60% of dementias and is a major public health burden. In Korea, the number of people with AD is estimated to be 705,473; this figure is predicted to reach 20 million by the year 2039. About 10% of the population over 65 years old are diagnosed with AD in Korea. The annual medical cost per person with dementia was estimated to be approximately 2,074 million Korean won (KRW). The national economic cost attributable to dementia, including direct and indirect medical costs, was estimated to be approximately 1.4 trillion KRW, accounting for 0.8% of the Korean GDP, and is projected to increase in the future.[1] Therefore, early

detection of AD and identification of modifiable risk factors are important for optimal disease intervention and management.

AD is histologically characterized by the presence of extracellular β-amyloid (Aβ) plaques and intracellular neurofibrillary tangles (NFTs). The amyloid hypothesis of AD proposes that an imbalance between Aβ production and clearance plays a central role in AD.[2,3] However, the validity of this theory has been challenged because no therapeutic interventions targeting Aβ have yielded promising results. In addition, emerging evidence shows that profound alterations in multiple cellular pathways begin long before the onset of clinical symptoms.[4-7]

Early-onset AD (EAOD), which presents in patients under the age of 65 years, affects less than 5% of the AD population and is known as a dominantly inherited familial disease in which genetic mutations related to amyloidogenic pathways have been identified. Conversely, late-onset AD (LOAD) accounts for the majority of cases and its incidence increases with age. LOAD is a rather complex and multifactorial disease that results from a combination of genetic, environmental, and lifestyle risk factors; hence, the amyloid cascade theory alone cannot fully explain all aspects of the disease pathogenesis.[8,9] This lack of understanding regarding the heterogeneity of AD could explain why previous clinical trials targeting Aβ have repeatedly failed to show clinical benefits.[9]

Owing to the failure of previous therapeutic interventions, there is an increasing demand to identify alternative mechanisms of AD pathogenesis. An alternative hypothesis proposes a link between AD and metabolic distress, which often precedes the development of AD. The presence of metabolic risk factors in midlife, such as hypertension, diabetes mellitus, arteriosclerosis, obesity, and hypercholesterolemia, are associated with cognitive decline later in life. In particular, cholesterol metabolism is linked to these metabolic risk factors.[10-12] The role of cholesterol in AD pathophysiology has been noted in studies of apolipoprotein E (ApoE), an important cholesterol transporter in the brain, which is strongly implicated in the risk for LOAD.[13]

2. Cholesterols in AD pathology

The brain is a cholesterol-rich organ that accounts for 2.1% of total body weight and 23% of total cholesterol content.[14] Cholesterol is a major component of neuronal membranes and plays an important role in the development and maintenance of neuronal function.[15] Cholesterol acts as a precursor for the synthesis of steroid hormones and oxysterols and regulates the activities of membrane-bound enzymes, receptors, and ion channels.[16] Cholesterol homeostasis is tightly regulated within the brain, and disturbed cholesterol metabolism may cause structural and functional impairments in the brain, which could potentially lead to AD.

Increased biologically active free cholesterol levels are associated with the presence of NFTs.[17] Increased cholesterol levels are associated with increased Aβ production in cell cultures,[18-20] and high-fat/high-cholesterol diet-induced hypercholesterolemia also significantly increases Aβ levels in the brain of transgenic mice.[21,22] Furthermore, a high cholesterol diet upregulates β-amyloid converting enzyme 1 (BACE1; β-secretase) through the SIRT1-PPARγ-PGC-1 pathway in C57BL/6 mice.[23]

To understand the relationship between cholesterol and Aβ production, understanding of the concept of lipid rafts is necessary. Lipid rafts are subdomains of the plasma membrane that are enriched in cholesterol and glycosphingolipids. Lipid rafts play important roles in neuronal cell functions including signal transduction, cell adhesion, and the trafficking and organization of bilayer components such as enzymes, receptors, and ion channels.[14,15,24] Importantly, amyloid precursor protein (APP), BACE1, and γ-secretases interact within the lipid rafts of neuronal cells. BACE1 and γ-secretase proteins are predominantly localized within the rafts, while the tumor necrosis factor alpha converting enzyme (TACE; α-secretase) and a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) are predominantly located outside of the rafts. Previous reports suggest that the amyloidogenic processing of APP occurs in lipid rafts, while the non-amyloidogenic processing occurs mainly in the non-raft regions.[25-27] Increasing membrane cholesterol levels augments lipid raft abundance, which

favors the generation of Aβ through the amyloidogenic pathway.[28]

Cholesterol metabolism regulates the amyloidogenic pathway and might play various roles in the brain with respect to AD pathogenesis. Recently, genes known as AD risk factors, such as clusterin (*CLU*), ATP binding cassette subfamily A member 7 (*ABCA7*), and sortilin related receptor 1 (*SORL1*), have also been reported to be strongly associated with cholesterol metabolism.[29-31] Moreover, cholesterol also affects tau pathology. Tau is a microtubule-associated protein that plays essential roles in determining neuronal structure and plasticity, and its functions are regulated by site-specific phosphorylation.[32] A high cholesterol diet has been reported to induce hyperphosphorylation of the tau protein in mice.[33] Further, dendrite outgrowth is reduced in cholesterol-deficient neurons because of decreased microtubule stability, owing to the inhibition of the phosphorylation of microtubule-associated protein 2 (MAP2), a cytoskeletal protein crucial for microtubule stability.[34] Therefore, cholesterol metabolism seems to play complex roles in the brain with regard to AD pathology.[35,36]

As cholesterol is linked to changes in AD pathology, studies have been performed to understand the relationship between cholesterol metabolism and AD development. The Washington Heights/Inwood Columbia Aging project showed that increased cholesterol (total cholesterol and LDL-C) levels and a history of diabetes were associated with faster cognitive decline in patients with AD.[37] In a cohort of 9,844 participants aged 40-45 years, midlife high serum total cholesterol was associated with increased risk for developing AD.[35] In other epidemiological studies, hypercholesterolemia in middle-aged individuals also correlated with the risk for developing AD.[38,39] However, other studies that particularly investigated the link between cholesterol levels and AD risk showed conflicting results.[12,40] There was no correlation between total cholesterol levels and AD development in the large population-based cohort from the Framingham and Honolulu-Asia aging study.[41] Nevertheless, in the elderly, it has even been reported that low total cholesterol levels are associated with cognitive decline. These contradicting results could be attributed to the limitation of simply measuring plasma cholesterol levels because serum total cholesterol levels do not reflect complex cholesterol metabolism.[42] In fact, cholesterol is

metabolized to various bioactive derivatives or metabolites. Therefore, AD-related pathology might be influenced by the distribution of cholesterol in different cell compartments and altered metabolism to various metabolites.[43-45]

3. Cholesterol metabolism and oxysterols

Although cholesterol metabolism is associated with AD pathogenesis,[46,47] a mechanism explaining the link between AD and cholesterol has not yet been established. The blood-brain barrier (BBB) prevents peripheral cholesterol from entering the brain; hence, brain cholesterol is largely independent of dietary intake and hepatic synthesis. The brain synthesizes its own cholesterol. Total cholesterol levels steadily increase between the ages of 20 and 65 years in humans.[48] The maintenance of cholesterol homeostasis is crucial for neuronal function and brain development. In cholesterol-fed rabbits, brain cholesterol remained unchanged while blood cholesterol levels were highly increased.[49] Instead, the administration of a high fat diet in an AD animal model was able to induce Aβ deposition and worsen cognitive functions by changing the flux of oxysterols to the brain.[50,51] Oxysterols differ from cholesterol in the presence of an additional polar moiety. Brain cholesterol has been suggested to maintain its levels and exert its functions through cholesterol metabolites. In light of this, oxysterols, oxidized derivatives of cholesterol, are currently the subject of interest in AD research because they can cross the BBB in both directions.[52]

Oxysterols are derived from both enzymatic and non-enzymatic pathways (Figure 1).[53] Their chemical structures vary according to the number and location of oxygenated functional groups including keto, hyroxy, and epoxy groups. Enzymatic pathways of oxysterol production mainly involve cytochrome P450 enzymes. For example, 27-hydroxylase (CYP27A1), a key enzyme for alternative bile acid synthesis, leads to the production of 27-hydroxycholesterol (27-OHC), while 7α-hydroxylase (CYP7A), a rate-limiting enzyme in the bile acid synthesis pathway, catalyzes the synthesis of 7α-

hydroxycholesterol.[54,55] Oxysterols that are produced by non-enzymatic pathways are generated through auto-oxidation. 7β-OHC is an example of an auto-oxidation product. The enzyme 24S-hyrdoxylase (CYP46A1) is only expressed in the brain. This enzyme converts excess cholesterol into 24-hydroxychoelsterol (24S-OHC), which is a more soluble and diffusible form of cholesterol that can easily cross the BBB. 24S-OHC levels are directly proportional to brain cholesterol levels.[56] Extracerebral cholesterol metabolites such as oxysterols also enter the brain via the circulation. 27-OHC is the most abundant cholesterol metabolite in plasma.[57] This metabolite efficiently regulates cholesterol synthesis, is transported via the peripheral circulation, and crosses the BBB by diffusion.[58] In non-pathological conditions, 27-OHC levels are significantly lower than 24-OHC levels in the brain due to the tight regulation of cholesterol homeostasis.[58,59]

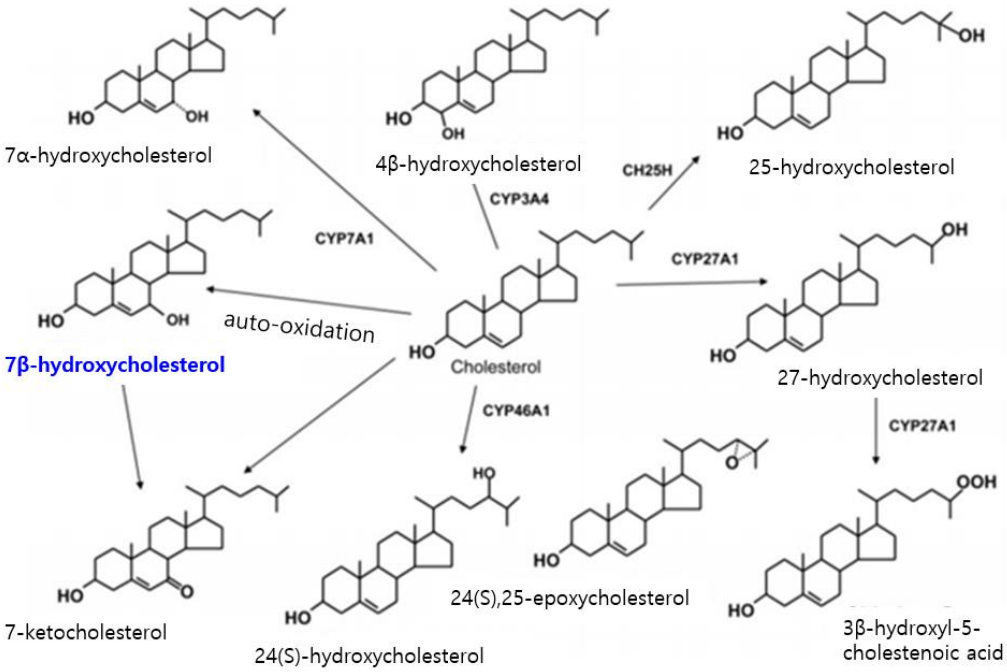

Figure 1. Structures of cholesterol and major oxysterols. Oxysterols are cholesterol oxidation products derived from autoxidation or enzymatic oxidation of cholesterol. The major oxysterols resulting from autoxidation (7β-hydroxycholesterol, 7-ketocholesterol) and enzymatic oxidation (24-hydroxycholesterol, 27-hyroxycholesterol, and 25-hydroxycholesterol) of cholesterol are presented.

Adapted from “Oxysterols and Their Cellular Effectors”, by Vesa M. Olkkonen and Eija Nissilä, Biomolecules 2012.[60]

4. Oxysterols in AD

The major oxysterols that have so far been associated with AD are 24S-OHC, 27-OHC, and 7β-OHC. CYP46A1 is the main enzyme regulating cholesterol elimination in the brain, and its levels are positively correlated with cognitive function.[61] Plasma levels of 24S-OHC were recently shown to be decreased in patients with advanced AD, while the levels of 24S-OHC in CSF were increased.[55,62,63] Since levels of ApoE, tau, and hyperphosphorylated tau (p-tau) are increased along with 24S-OHC in the CSF of AD patients, 24S-OHC is considered a sensitive marker for diagnosis of AD.[64] The levels of 27-OHC are significantly higher in homogenates of the temporal cortices of AD patients.[65] In vitro studies have shown that 27-OHC increases APP, Aβ, and phosphorylated tau levels,[66,67] and knocking out *CYP27A1* in mice improves the learning and memory deficits induced by a high-fat diet.[68] It appears that 27-OHC may mediate the negative effects of cholesterol on memory. Most studies have focused on either 24S- or 27-OHC, while few have focused on 7β-OHC.[69,70] In addition, in most of the studies, 24S- and 27-OHC were measured in the CSF of participants, making it difficult to use these as early diagnostic markers for AD in large-scale population-based screening. The collection of CSF through lumbar puncture is an invasive procedure with potential complications. To examine the role of cholesterol metabolites in AD pathology, novel, convenient, and safe methods are needed.

5. Hair cholesterol profiling

The skin, which is the largest organ of the body, can act as a peripheral neuroendocrine organ that is closely linked to central stress.[71] As an adnexal structure of the skin, the hair can reflect systemic cortisol exposure over longer periods of time.[72] Compared to blood and CSF samples, hair fiber is very stable

and easy to collect and store.[73] Analytic techniques for hair cholesterol derivatives have recently been developed. Scalp hair has proven useful in identifying endocrine disorders, and several studies have reported the benefits of hair-based metabolomic profiling in stress-mediated chronic diseases.[74-76] It has been suggested that the levels of steroids in the hair can reflect chronic exposure to systemic steroids more reliably than their one-time plasma levels, which can easily be influenced by diurnal variation or diet.[77-79]

6. Unexplored role of 7β-OHC in AD

7β-OHC is a derivative of cholesterol oxidation via a non-enzymatic pathway, and it was initially associated with the pathogenesis of atherosclerosis. This oxysterol has been identified in atheromatous plaques and associated with endothelial cell dysfunction. Impairment of vascular homeostasis by 7β-OHC has been shown to induce an inflammatory phenotype in endothelial cells, and 7β-OHC is involved in oxLDL-induced monocyte differentiation, which is an essential prerequisite for fibrous cap formation.[80,81] In smooth muscle cells, 7β-OHC disrupts intracellular Ca^{2+} homeostasis and activates mitogen-activated protein kinase.[82] It also induces oxidative stress and apoptosis of human cells.[83,84]

The formation of 7β-OHC has recently been associated with AD. The levels of 7β-OHC are increased in the plasma,[85,86] frontal cortex, and CSF of AD patients.[87,88] 7β-OHC is known to be highly neurotoxic,[89] and its production via cholesterol oxidation is induced by APP and Aβ.[90] However, the direct role of 7β-OHC in AD pathogenesis remains largely unexplored. It is unclear whether the increase in 7β-OHC levels is a consequence of neurodegenerative processes or whether 7β-OHC plays a role in promoting AD pathogenesis. There have been no extensive investigations regarding whether the levels of 7β-OHC in the scalp hair are associated with AD, and if so, whether 7β-OHC has a specific role in AD pathogenesis.

Therefore, the aim of the present study was to investigate the relationship between 7β-OHC and AD, and the role of 7β-OHC in AD pathophysiology. First, I measured hair 7β-OHC levels in participants with normal cognition (NC), mild cognitive impairment (MCI), and AD, and investigated their

relationships with clinical parameters of AD. Second, I performed *in vivo* studies to examine whether 7β-OHC directly affects AD-related pathologies and cognitive behavior. Herein, I report that hair 7β-OHC levels are significantly increased in AD patients than in those with normal cognition and that 7β-OHC induces AD pathologies in animal models, suggesting its potential role as a biomarker for AD pathogenesis.

II. MATERIALS AND METHODS

1. Chemicals and reagents

Reference standards of 19 sterols were obtained from Sigma (St. Louis, MO, USA) and Steraloids (Newport, RI, USA). Other chemicals, including the deuterium-labeled internal standards (ISs) 2,2,3,4,4,6-d_6-cholesterol for free cholesterol and three plant sterols; 2,2,3,4,4,6-d_6-cholesteryl stearate (C/D/N Isotopes, Pointe-Claire, Quebec, Canada) for two cholesterol esters (CEs); and 25,26,26,26,27,27,27-d_7-4β-hydroxycholesterol and 25,26,26,26,27,27-d_6-27-hydroxycholesterol (Avanti Polar Lipids, Alabaster, AL, USA) for four cholesterol precursors and nine hydroxycholesterols (OHCs), were obtained. The trimethylsilyl (TMS) derivatizing agents *N*-methyl-*N*-trifluorotrimethylsilyl acetamide (MSTFA), ammonium iodide (NH_4I), and dithioerythritol (DTE), were purchased from Sigma. Hybrid solid-phase extraction/precipitation cartridge (H-PPT; 1 mL, 30 mg) was supplied by Supelco (Bellefonte, PA, USA). All organic solvents were of analytical and high-performance liquid chromatography grades and were purchased from Burdick & Jackson (Muskegon, MI, USA). The pulverization of hair samples was performed using a tissue lyser (Qiagen, Hilden, Germany). 7β-OHC was purchased from Sigma (H6891, MW 402.65).

2. Hair metabolic profiling of cholesterols

Quantitative metabolite profiling of hair cholesterols was performed using gas chromatography-mass spectrometry (GC-MS).[91] The stability of the analyst during sample collection and handling has already been validated in previous reports.[78] Two strands of hair were obtained from the vertex of participants' scalps and were then pulverized in 0.5 mL of methanol/dichloromethane (1:2) solution with three zirconia beads using the TissueLyser at 25 Hz for 10 minutes. This pulverization process was then repeated. Supernatants were discarded after centrifugation (12,000 rpm, 5 minutes). Sterol-free hair samples were used for calibration and quality control (QC) purposes. Each hair sample was filtered and washed three times with methanol/dichloromethane (1:2) solution and dried at 60 °C for 1 hour. Sterol-free hair was obtained as determined by the negative results for all analytes.

3. GC-MS analysis

GC–MS was performed using an Agilent 6890 Plus gas chromatograph interfaced with a single-quadrupole Agilent 5975C MSD (Agilent Technologies, Palo Alto, CA, USA). Each sample (2 μL) was injected in split mode (10:1) at 300 ºC and separated through an MXT-5 capillary column (15 m × 0.25 mm inner diameter, 0.25 μm film thickness, Silcosteel-treated stainless steel; Restek; Bellefonte, PA, USA). Oven temperature was initially maintained at 265 ºC for 5 minutes, then increased to 280 ºC at 2 ºC/minute, and finally increased to 380 ºC at 5 ºC/minute (held for 3 minutes). The carrier gas was ultra-high-purity helium at a column head pressure of 89.6 kPa (13 psi; column flow: 1.1 mL/minute at an oven temperature of 265 ºC).

4. Participants

Participants were classified into three groups according to their cognitive status: NC, MCI, and AD. Of the 285 participants recruited from a memory clinic of a university-affiliated general hospital (Seoul, South Korea) and a community dementia center, 82 participants with NC, 39 with MCI, and 81 with

AD were finally selected (Figure 2). The demographics of the participants are described in Table 1. This study was approved by the Institutional Review Board of Severance Hospital at Yonsei University Health System. Exclusion criteria were: (1) a history of major psychiatric illness; (2) a history of major neurological illness such as stroke, head trauma, or epilepsy; (3) other dementias including vascular dementia, Lewy body disease, and frontotemporal dementia; (4) being younger than 60 years or older than 80 years old; and (5) having a Global Deterioration Scale rating (GDS) > 5. All participants were evaluated and diagnosed by two board-certified psychiatrists based on a clinical interview, neuropsychological tests, and blood tests. The diagnostic classification of AD was based on the Diagnostic and Statistical Manual of Mental Disorders[92] and that of MCI was based on Peterson's criteria.[93] The Mini-Mental State Examination (MMSE) and GDS were used to measure cognitive ability and severity of dementia. Two strands of hair were obtained from the vertex of participants' scalps for quantitative sterol analysis. Age, sex, and years of education did not significantly differ between the groups (Table 1).

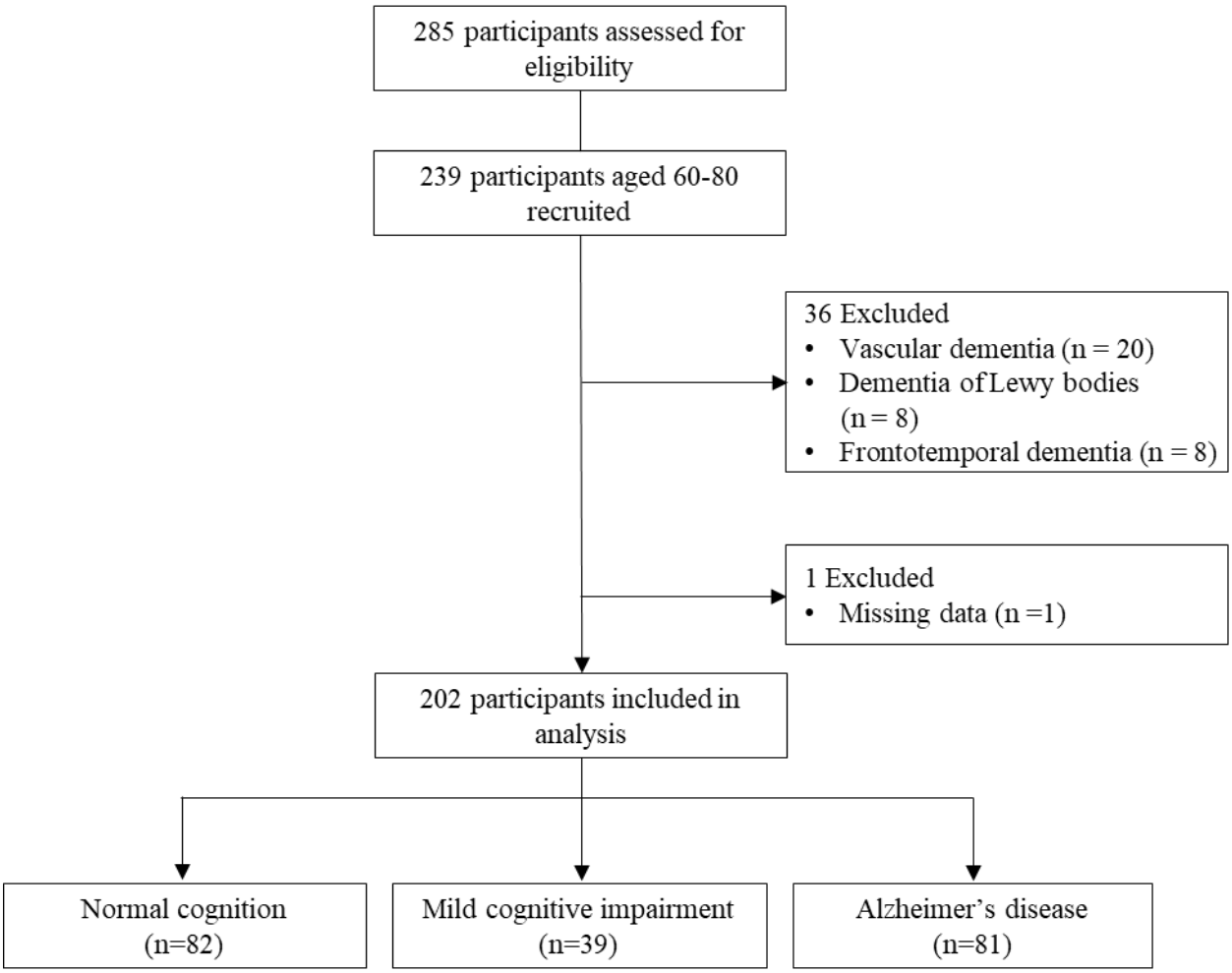

Figure 2. Flow chart of study participants. Two hundred eighty-five participants from a memory clinic of a university-affiliated general hospital and a community dementia center were assessed for eligibility, and 239 participants aged between 60 and 80 years were finally recruited. Among them, 20 patients with vascular dementia, 8 patients with dementia with Lewy bodies, and 8 patients with frontotemporal dementia were excluded. A total of 202 patients were included in the analysis—82 participants with normal cognition, 39 with mild cognitive impairment, and 81 with Alzheimer's disease.

Table 1. Participant demographics

Variable		**NC (n = 82)**	**MCI (n = 39)**	**AD (n = 81)**	***p***
Age (years)		71.1 (5.12)	72.7 (5.71)	72.2 (5.23)	0.257
Female patients, n (%)		52 (63.4%)	29 (74.4%)	59 (72.8.0%)	0.323
Education (years)		7.9 (4.88)	7.7 (4.91)	8.3 (3.87)	0.766
MMSE		27.6 (1.61)	25.3 (2.52)	21.3 (4.09)	<0.001
GDS	1	59 (72%)	-	-	<0.001
	2	23 (28%)	28 (71.8%)	2 (2.5%)	
	3	-	11 (28.2%)	56 (69.1%)	
	4	-	-	14 (17.3%)	
	5	-	-	9 (11.1%)	
Cholesterol (mg/dL)		190.7 (30.28)	188.7 (44.05)	180.3 (44.36)	0.761
HDLc (mg/dL)		68.0 (31.19)	46.8 (10.40)	49.1 (13.31)	0.065
LDLc (mg/dL)		98.9 (41.02)	115.3 (40.54)	108.4 (38.66)	0.768
Triglyceride (mg/dL)		118.7 (38.89)	115.7 (37.63)	131.5 (71.34)	0.724
Dyslipidemia, n (%)		29 (35.8%)	15 (38.5%)	22 (27.2%)	0.360
Statin use, n (%)		26 (31.7%)	12 (30.8%)	21 (25.9%)	0.702
Hypertension, n (%)		50 (61.0%)	25 (64.1%)	39 (48.1%)	0.145
Diabetes mellitus, n (%)		22 (26.8%)	5 (12.8%)	20 (24.7%)	0.219

Data are presented as mean (SD), n (%). One-way analysis of variance (ANOVA) was used to compare between diagnosis states. Abbreviations: NC, normal cognition; MCI, mild cognitive impairment; AD, Alzheimer's disease; HDLc, high density lipoprotein cholesterol; LDLc, low density lipoprotein cholesterol. MMSE, Mini-Mental State Examination; GDS, Global Deterioration Scale.

5. Animals and treatment

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Yonsei University Health System, in accordance with the guidelines of the Animal Welfare Act. A total of 39 male C57BL/6 mice were obtained from Orient Bio (Orient Bio Inc., Seongnam, Korea) and bred at Yonsei University College of Medicine. All mice were aged between 16 and 18 weeks at the onset of the experiment. Seventeen mice were assigned for biological study, and they were randomly assigned to either the vehicle (n = 8) or 7β-OHC group (n = 9). In addition, 22 mice were assigned for further behavioral experiments. All mice participating in the study were housed under a 12-hour light/dark cycle with food and water available ad libitum. They were anesthetized with a mixture of nitrogen gas and oxygen gas and placed on a stereotaxic apparatus (JD-SI-02, JEUNG-DO B&P Co., Ltd., Seoul, South Korea). 7β-OHC (50 μM dissolved in 0.1% ethanol in PBS) or vehicle (0.1% ethanol in PBS) were stereotaxically injected into the bilateral hippocampus through a small craniotomy using the following coordinates with respect to the bregma: anterior-posterior (AP): -2.00 mm, medial-lateral (ML): ±1.3 mm, and dorsal-ventral (DV): -2.2 mm. The needle was withdrawn 5 minutes after the hippocampal injection, and the scalp was sutured. An intracerebroventricular infusion was conducted for the behavioral experiment to minimize the effects of additional behavioral changes due to tissue damage. The mice were sacrificed 3 days after the injection (Figure 3). The right cortex and hippocampus were immediately harvested, placed on ice, and stored in a freezer at -80°C until western blot and ELISA analyses were conducted. For immunohistochemistry analysis, the left-brain hemisphere was immediately fixed after harvesting, using a 3.7% formaldehyde-containing phosphate-buffered saline (PBS). It took less than 8 minutes from decapitation to tissue-freezing for all mice.

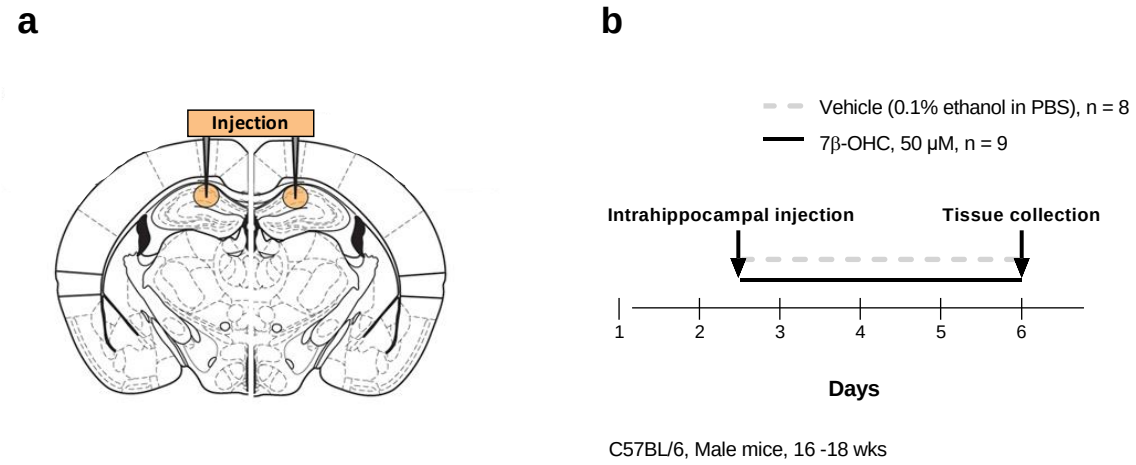

Figure 3. General scheme of the experiment. (a) Hippocampal injection of 7β-OHC in C57BL/6 mice. The drawings were adapted from the "Mouse Brain in Stereotaxic Coordinates Atlas".[94] (b) Experimental scheme for the stereotaxic injection and tissue collection. Animals were sacrificed 3 days after stereotaxic injection. Abbreviations: 7β-OHC, 7β-hydroxycholesterol.

6. Immunohistochemistry

The fixed, frozen left hemispheres were cut into 20-μm coronal sections using a cryostat. Sections were stained whilst floating. They were first permeabilized in PBS containing 0.3% Triton X-100 for 1 hour. Sections were then blocked using 5% bovine serum albumin (BSA) in PBS containing 0.3% Triton X-100 for 1 hour. After blocking, sections were incubated with anti-GFAP-antibody (1:500, ab173004, Synaptic Systems, Göttingen, Germany) and anti-Iba1-antibody (1:100, 019-19741, Wako, Osaka, Japan) in PBS containing 2% BSA and 0.3% Triton X-100 at 4 °C overnight. The slices were then washed three times with PBS and labeled with secondary antibodies (1:500) targeting donkey anti-goat IgG conjugated to Alexa-488 (705-545-003, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 hour. The sections were then washed three times with PBS and counter-stained with 4, 6-diamidino-2-phenylindole (DAPI) (D9542, Sigma-Aldrich, St. Louis, MO, USA) and observed under a confocal laser scanning microscope (Carl Zeiss, Thornwood, NY, USA).

7. Cytokine production assay

The levels of interleukin (IL)-1β and IL-6 in mouse brain homogenates were quantified using mouse IL-1β (ab46052; Abcam, Cambridge, MA, USA) and IL-6 ELISA kits (M6000B; R&D Systems, Minneapolis, MN, USA). All experiments were performed according to the respective manufacturer's protocols.

8. Cell viability assay

Apoptotic nuclear DNA breaks in the tissue sections were assessed by double-fluorescent labeling using a terminal dUTP nick-end labeling (TUNEL) assay (11684795910; Sigma-Aldrich, St. Louis, MO, USA). The brain hemisphere sections (20-μm thick) were air-dried at room temperature and

permeabilized in PBS containing 0.3% Triton X-100 for 1 hour. Positive control sections were incubated with 20 μg/mL proteinase K solution for 30 minutes. Then, the sections were incubated with reaction mixtures for 60 minutes at 37 °C in a dark, humidified atmosphere. The sections were washed three times with PBS and counter-stained with DAPI.

9. Western blot analyses

Brain tissues from the right hemisphere were lysed in a buffer containing 20 mM HEPES (pH 7.0), 1 mM EGTA, 10 mM KCl, 1.5 mM $MgCl_2$, 250mM sucrose, and protease inhibitors. Soluble lysates were fractionated by SDS–polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes. The following antibodies were used for detecting BACE1 (1:1,000, 5606S; Cell Signaling Technology, Beverly, MA, USA), TACE (1:1,000, sc-13973; Santa Cruz Biotechnology, Dallas, TX, USA), APP (1:1,000, 2450S; Cell Signaling Technology), sAPPβ (1:1,000, sig-39138; Biolegend, San Diego, CA, USA), Aβ (1:1,000, sc-28365; Santa Cruz Biotechnology), tumor necrosis factor-α (TNF-α; 1:1,000, sc-133192; Santa Cruz Biotechnology), phospho-tau (Ser262; 1:1,000, sc-101813; Santa Cruz Biotechnology), phospho-tau (Ser199, Ser202; 1:1,000, 44-768G; Invitrogen, Carlsbad, CA, USA), phospho-tau 356 (1:1,000, sc-101814; Santa Cruz Biotechnology), total tau (1:1,000, 4019; Cell Signaling Technology), and GAPDH (1:20,000, sc-25778; Santa Cruz Biotechnology). Signal intensity was measured using Multi Gauge 3.0 analysis software (Fujifilm, Tokyo, Japan).

10. Touchscreen-based behavior experiment

A. Apparatus

Cognitive functions were assessed by the Bussey-Saksida mouse touchscreen operant system

(Campden Instruments Ltd., Loughborough, UK) as previously reported.[95-97] A trapezoidal touchscreen operant chamber was housed in a sound- and light- attenuating box (Figure 4). A fan was fitted in the box to improve ventilation and block outside noise. The chamber consisted of a touchscreen (12.1-inch, resolution of 800 × 600) which presented the stimulus. Undesirable responses were blocked, and different spatial locations were created by placing a black Perspex mask in front of the touchscreen. In this experiment, a 5-window mask was used (Campden Instruments, Ltd.). A magazine (reward dispenser) was placed next to a 3 W light bulb on the opposite side of the touchscreen. During each trial of the experiment, 20 μL of reward was delivered to the magazine, and the light was illuminated. Infrared beams were installed near the magazine and touchscreen to detect the motor activity of the mice. All behavioral procedures were controlled and implemented by ABET II Touch software (Campden Instruments, Ltd.) and Whisker Server.[98]

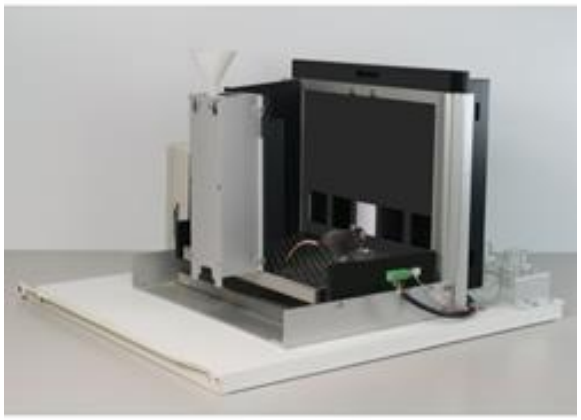

Figure 4. The 5-choice serial reaction time (5-CSRT) task in touchscreen chambers for mice. This task measures attentional ability, requiring mice to respond to a brief visual stimulus presented randomly in one of five locations.

B. Acquisition

Attention and response control ability were assessed by a 5-choice serial reaction time (5-CSRT) task. The experiment was conducted as previously described.[99] Briefly, the mice were trained to initiate

each trial by entering the magazine and touching the stimulus on the touchscreen to get the reward. During acquisition, the mice were trained to respond to the pseudo-randomly located stimulus within a stimulus duration (SD) and limited hold (LH). The stimulus was presented on the screen only during the SD, and mouse responses were recognized for a further 5 seconds of LH. Each session was terminated when the mice completed 60 trials or when 1 hour had elapsed. When all mice achieved an accuracy rate greater than 80%, the baseline was set with an SD of 2 seconds for 2 consecutive days. During this acquisition phase, two mice were excluded because they did not meet the accuracy requirement.

C. Probe test

After 1 week of recovery from the stereotaxic infusion surgery, the mice underwent a reminder session. They all met the requirement; hence, they were all included in the probe session. Probe sessions were conducted for 4 consecutive days. The SDs were 2, 1.5, 1, 0.5 seconds to tax the attentional load. Stimuli were pseudo-randomly presented at one of five locations.

11. Statistical analysis

Statistical analyses were conducted using the Statistical Package for the Social Sciences version 25.0 (SPSS, Inc., Chicago, IL, USA), R Statistical Software version 3.0.2 (R Foundation for Statistical Computing, Vienna, Austria), and GraphPad Prism software version 8.02 (San Diego, CA, USA). Non-normally distributed data were transformed into natural logarithms for statistical analysis. Between-group differences were analyzed using analyses of variance (ANOVA) or analyses of covariance (ANCOVA) with Bonferroni post hoc tests. Age, sex, education, and statin usage were used as covariates in the ANCOVA test. The Kruskal-Wallis test was used to determine the between-group differences in hair sterol levels. To test the correlations between hair cholesterol level and the MMSE

and GDS scores, partial correlation analysis was performed with age, sex, education, and statin usage as covariates. In the animal study, unpaired two-tailed *t*-test was used to analyze the statistical difference between the vehicle and 7β-OHC groups. Continuous variables are expressed as means ± standard deviation or median (interquartile range, IOR), and categorical variables are expressed as quantities and percentages. Statistical significance was set to $\alpha = 0.05$.

III. RESULTS

1. 7-OHCs levels in the hair of AD patients

Of the 19 sterols monitored, 9 were quantitatively detected in two strands of 3-cm-long hair samples (Table 2). All the groups had similar levels of absolute free cholesterol, cholesterol esters, and cholesterol precursors, while the AD group had significantly higher sitosterol levels. Both 7α-OHC and 7β-OHC levels were significantly higher in those with MCI and AD than in those with NC. Post-hoc comparisons showed a marginal difference in 7α-OHC levels between participants with NC and AD ($p = 0.057$). 7β-OHC levels were significantly different between participants with NC and AD ($p = 0.004$).

Table 2. Sterol levels in the scalp hair

Compounds	NC			MCI			AD			*p**			
	N	Median	IQR	N	Median	IQR	N	Median	IQR	Overall	NC vs. MCI	NC vs. AD	MCI vs. AD
Free cholesterol	79	609.5	302.2	39	522.4	303.4	78	610.45	262.5	0.428	> 0.999	> 0.999	0.583
Cholesteryl laurate	74	1.5	1.9	37	1.2	1	76	1.5	1.35	0.297	0.358	>0.999	0.912
Cholesterlyl myristate	76	21.1	12.75	37	22	13.4	73	24.7	12.4	0.249	> 0.999	0.373	0.66
Sitosterol	78	1.05	0.7	39	1.2	0.8	77	1.3	0.9	0.002	0.439	0.001	0.443
Desmosterol	79	103.5	48.3	39	97.9	59.6	78	98.75	60	0.469	0.664	> 0.999	> 0.999
Lathosterol	79	10	5.9	39	7.6	3.7	78	9.45	5.7	0.175	0.208	> 0.999	0.376
Lanosterol	79	3.1	1.1	39	3.2	1.4	78	3.35	1.5	0.298	> 0.999	0.359	> 0.999
7α-Hydroxycholesterol	79	1.7	1.1	39	2.1	1.4	78	2.1	1.3	0.034	0.145	0.057	> 0.999
7β-Hydroxycholesterol	79	2.5	1	39	2.7	0.8	78	2.85	0.9	0.005	0.272	0.004	> 0.999

The statistical significance of group differences in cholesterol metabolite levels was determined by the non-parametric Kruskal-Wallis test. All values are given as median (interquartile range). Dunn's multiple comparison test was conducted for post hoc analysis of all variables.

* $p < 0.05$ for significant differences. Abbreviations: NC, normal cognition; MCI, mild cognitive impairment; AD, Alzheimer’s disease.

2. Relationship between 7-OHCs and AD severity

7β-OHC can vary significantly according to statin regimen[100]; hence, statin use was included as a covariate in addition to age, sex, and education level. A one-way ANCOVA test revealed a significant between-group difference in 7α-OHC (F [2,189] = 4.27, p = 0.015) and 7β-OHC (F [2,189] = 7.07, p = 0.001) levels (Figure 5a, 5d). Bonferroni post hoc test for multiple comparisons showed that the AD group had significantly higher 7α-OHC and 7β-OHC levels than the NC group. Based on these findings, the correlation of hair cholesterol levels with cognition and dementia severity was calculated. A significant correlation was found between 7β-OHC levels and MMSE and GDS scores (Figure 5b, 5c). A significant correlation was also found between 7α-OHC levels and GDS scores (Figure 5f), but no correlation was found between 7α-OHC levels and MMSE scores (Figure 5e).

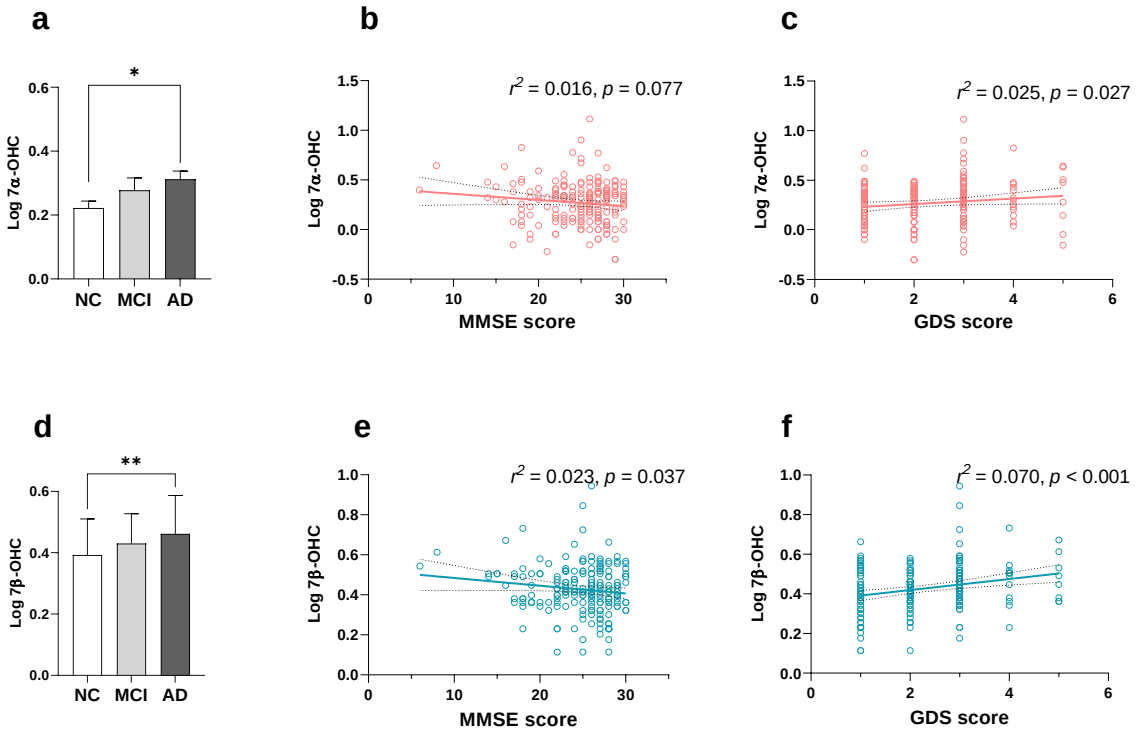

Figure 5. Relationship between hair 7α/β-OHC levels and AD severity and clinical features. Partial correlation coefficients (r^2) adjusted for age, sex, education, and statin use were calculated to show the relationship between oxysterols and clinical features of AD. (a) Hair 7α-OHC levels increased according to dementia severity. Post hoc comparisons indicated a significant difference between NC and AD groups (p = 0.013). (b) There was no correlation between 7α-OHC levels and MMSE scores. (c) 7α-OHC and GDS scores were positively correlated. (d) Hair 7β -OHC levels increased according to dementia severity. Post hoc comparison identified a significant difference in 7β -OHC levels between NC and AD (p = 0.001). (e, f) There was a significant positive correlation between 7β-OHC levels and both MMSE and GDS scores. *p < 0.05 and **p < 0.01 in post hoc comparisons. Abbreviations: OHC, hydroxycholesterol; NC, normal cognition; MCI, mild cognitive impairment; AD, Alzheimer's disease; MMSE, Mini-Mental State Examination; GDS, Global Deterioration Scale.

3. 7β-OHC levels and increased risk of AD

Since I found that both 7α-OHC and 7β-OHC are closely related to AD, I conducted logistic regression analysis to determine the association between AD risk and the levels of 7α-OHC and 7β-OHC. Table 3 shows the age-, sex-, education-, and statin usage-adjusted risk estimation for AD for 7α-OHC and 7β-OHC. 7β-OHC levels were associated with both AD risk (adjusted OR = 1.55) and cognitive impairment, which accounts for both MCI and AD (adjusted OR = 1.56). However, there was no statistically significant correlation between 7α-OHC levels and either AD or cognitive impairment.

Table 3. Binary logistic regression analysis of 7-oxysterols and AD risk

		OR	**95% CI**	***p***
Cognitive impairment (MCI+AD)	7α-OHC	0.99	0.801-1.231	0.95
	7β-OHC	1.56	1.036-2.342	0.03*
AD	7α-OHC	1.04	0.839-1.284	0.73
	7β-OHC	1.55	1.045-2.311	0.03*

*$p < 0.05$. Abbreviations: OHC, hydroxycholesterol; MCI, mild cognitive impairment; AD, Alzheimer's disease; CI, confidence interval.

4. Glial cell activation and pro-inflammatory cytokine production by 7β-OHC

Given that 7β-OHC levels were found to be correlated with higher odds of AD, I investigated whether 7β-OHC directly affects AD pathology such as neuroinflammation, Aβ levels, and tau phosphorylation. To observe the effect of 7β-OHC on the inflammatory response, 7β-OHC was stereotaxically injected into the mouse hippocampus, and the morphological changes of glial cells were observed (Figure 6a). GFAP intensity was increased in the CA1 and DG regions after injection of 7β-OHC (Figure 6b). Significant increase of Iba-1 positive microglia was observed at CA1 regions of 7β-OHC-treated mice (Figure 6c).

Activation of microglia by injection of 7β-OHC may stimulate the expression of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α.[101,102] I measured pro-inflammatory cytokines in brain homogenates, and found that IL-6 and IL-1β levels were significantly higher after injection of 7β-OHC (Figure 7), but TNF-α levels were not significantly changed (Figure 8). These results indicate that 7β-OHC induces neuroinflammation in the hippocampus. When cells die in vivo, they cause a variety of inflammatory responses.[103] Since 7β-OHC is known to be cytotoxic and can trigger apoptosis,[104] I examined whether increased neuroinflammation after 7β-OHC treatment is associated with apoptotic cell death. However, TUNEL staining showed no significant cell death in 20 μm of embedded frozen brain sections from 7β-OHC-treated mice compared to that in vehicle treated mice (Figure 9). These results suggest that the increase in neuroinflammation after 7β-OHC treatment was not due to cell death.

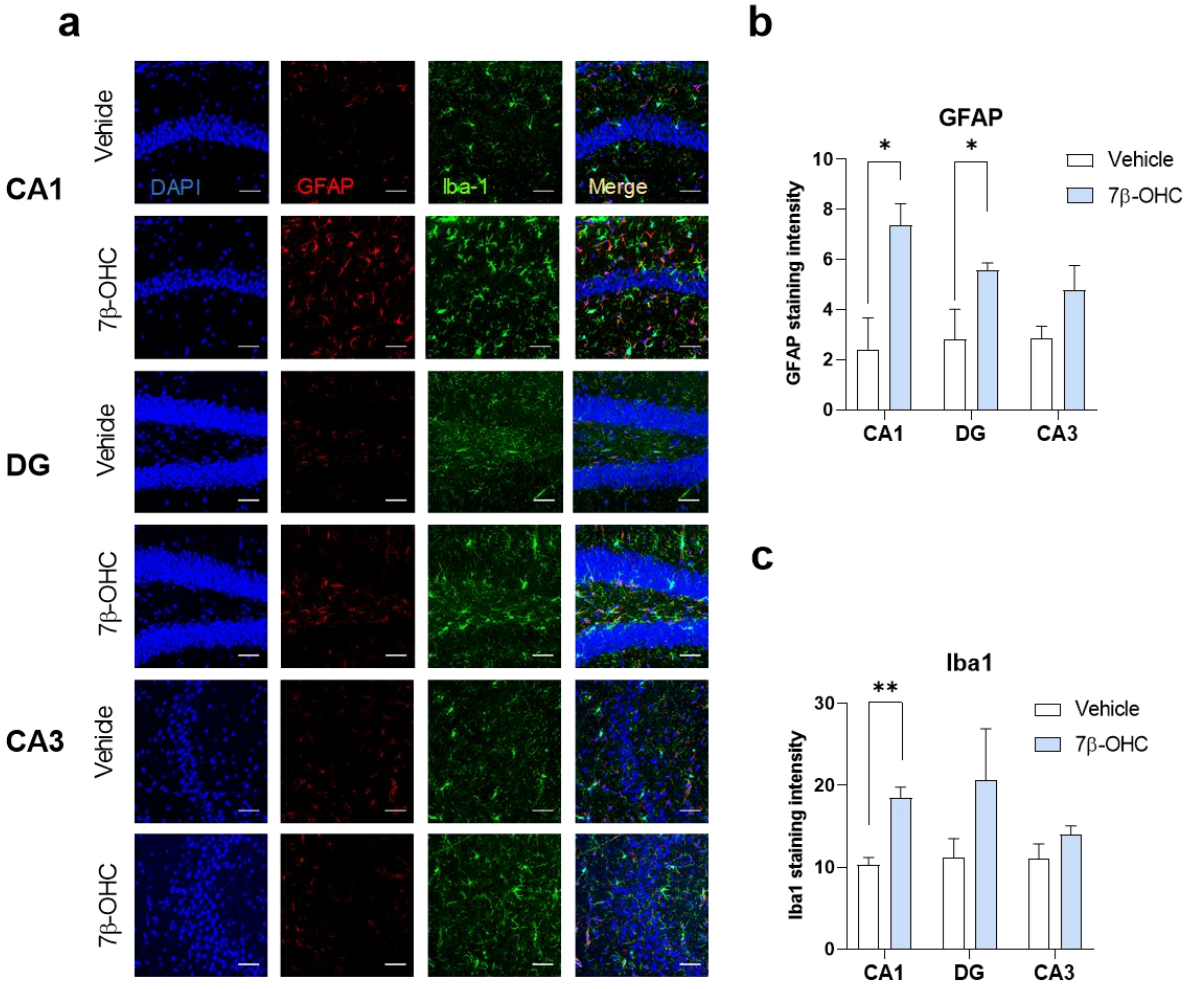

Figure 6. Glial activation upon 7β-OHC injection. (a) Immunohistochemical staining of the brain sections with anti-Iba-1 antibody (green), DAPI (Blue), and anti-GFAP antibody (red) after bilateral stereotaxic injection of vehicle (ethanol) or 7β-OHC (50 μM). (b) 7β-OHC-injected mice showed marked expression of GFAP in the CA1 and DG areas of the hippocampus. (c) Quantification of Iba1+ microglia in confocal images of vehicle- or 7β-OHC-injected C57BL/6 mice hippocampi (n = 5). *$p < 0.05$, **$p < 0.01$. Abbreviations: 7β-OHC, 7β-hydroxycholesterol; CA1, cornu amonis 1; DG, dentate gyrus; CA3, cornu amonis 3.

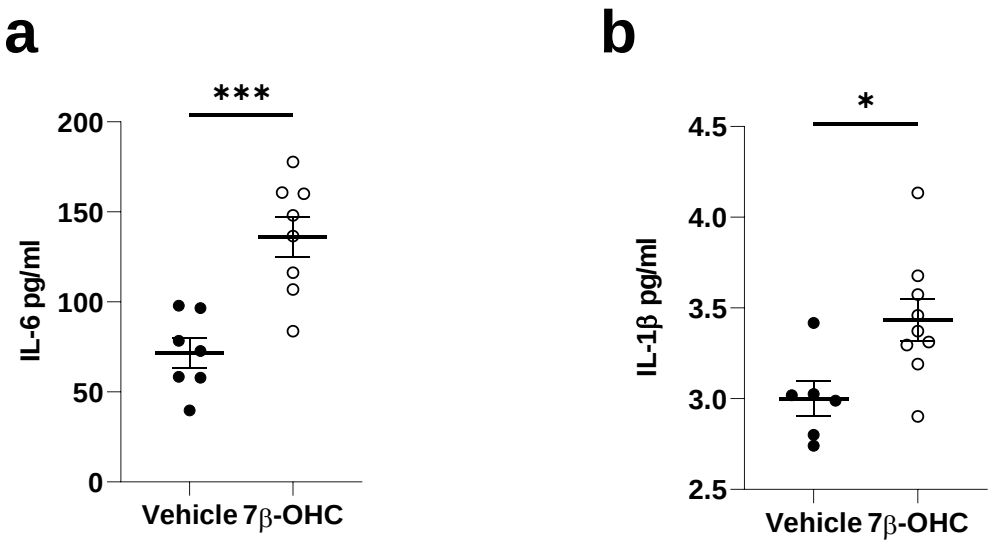

Figure 7. Pro-inflammatory cytokine levels measured by ELISA in the hippocampus of 7β-OHC-injected mice. (a) IL-6 protein levels were significantly elevated in the brain of 7β-OHC-injected mice. (b) Total IL-1β protein levels were elevated in the brain of 7β-OHC-injected mice. *p < 0.05, ***p < 0.001. Abbreviations: IL, interleukin; 7β-OHC, 7β-hydroxycholesterol.

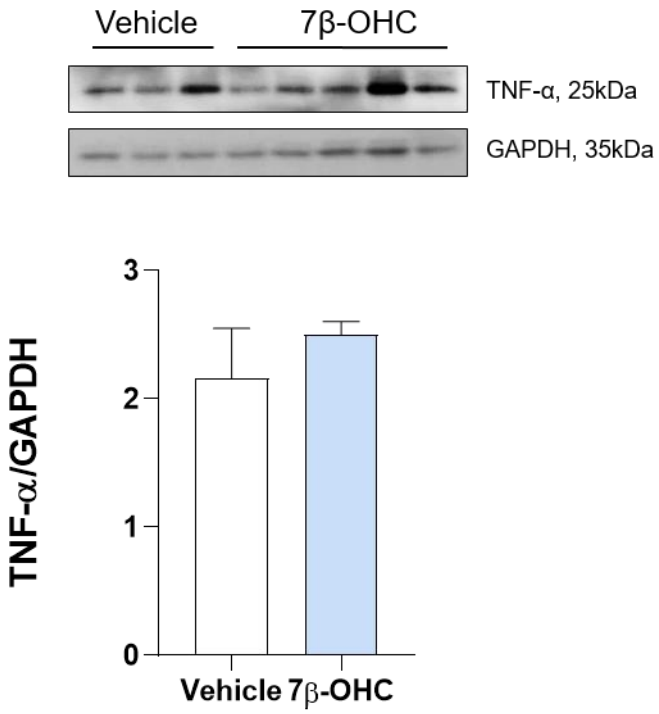

Figure 8. Expression levels of TNF-α in the homogenates of mouse hippocampus after 3 days of 7β-OHC treatment in comparison with those after vehicle treatment (n = 6 per group). The intensity of target bands was quantified using Multigage software and normalized to GAPDH levels. Abbreviations: 7β-OHC, 7β-hydroxycholesterol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TNF, tumor necrosis factor.

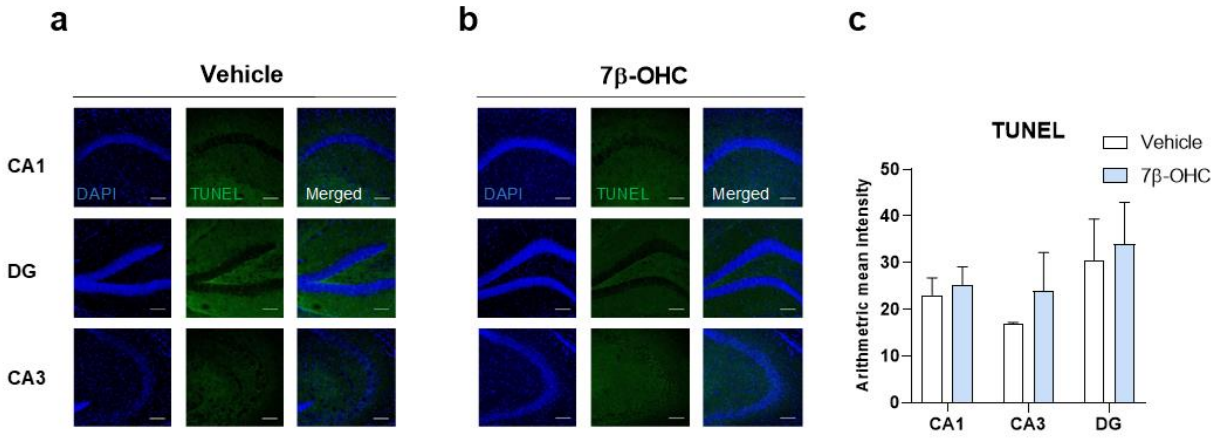

Figure 9. TUNEL-positive cell death was not observed after 7β-OHC treatment. (a, b) Detection of apoptotic cell death by the TUNEL assay (green) in CA1, DG, and CA3 regions of the hippocampus. DAPI (blue) was used for nuclear counterstaining. (c) Arithmetic mean intensity for TUNEL was measured using ImageJ software. Scale bar is 50 μm. Abbreviations: 7β-OHC, 7β-hydroxycholesterol; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling; CA1, cornu amonis 1; DG, dentate gyrus; CA3, cornu amonis 3.

5. 7β-OHC and BACE1 levels

Elevated neuroinflammation after 7β-OHC-treatment indicated that 7β-OHC could be involved in AD pathogenesis. Since Aβ can induce the oxidation of cholesterol to form 7β-OHC,[90] I hypothesized that 7β-OHC induces the activation of the amyloidogenic pathway. To test this hypothesis, I examined the effect of 7β-OHC on the levels of APP, TACE, and BACE1 in the mice. The levels of BACE1 increased significantly in 7β-OHC-treated mice while those of TACE decreased significantly (Figure 10a). Consistently, Aβ levels were increased (Figure 10b). Sirtuin 1 (SIRT1) levels were decreased after 7β-OHC treatment, and there were no significant changes in ADAM10 or sAPPβ levels. These results suggest that 7β-OHC stimulates the amyloidogenic pathway by upregulating BACE1 and down-regulating TACE expression.

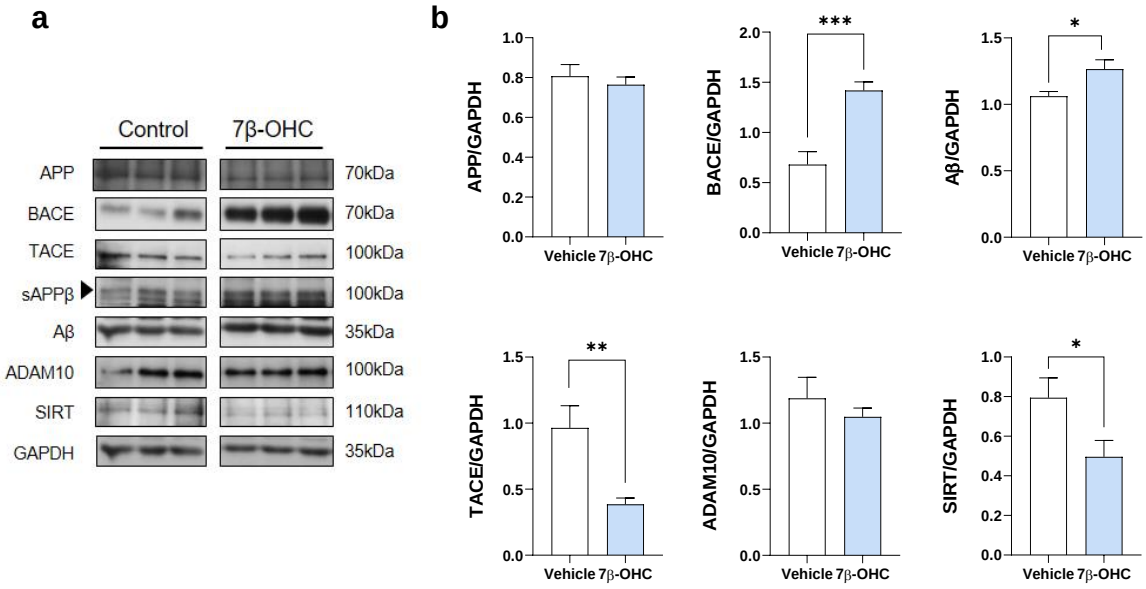

Figure 10. Effect of 7β-OHC on APP metabolism and production of Aβ. (a) Representative western blot analysis and (b) densitometry graph of proteins detected in the homogenates of mouse hippocampus after 3 days of 7β-OHC treatment compared with those after vehicle treatment (n = 6 per group). GAPDH was used as a loading control. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Abbreviations: Aβ, amyloid β; 7β-OHC, 7β-hydroxycholesterol; GAPDH, glyceraldehyde-3-phosphate

dehydrogenase; APP, amyloid precursor protein; BACE1, β-amyloid converting enzyme 1; TACE, tumor necrosis factor alpha converting enzyme; ADAM10, A disintegrin and metalloproteinase domain-containing protein 10; SIRT, sirtuin (silent mating type information regulation 2 homolog).

6. Tau phosphorylation upon 7β-OHC treatment

Tau phosphorylation at Ser262 (p-tau262), Ser396 (p-tau396), Ser199/202 (p-tau199/202), and Thr231 (p-tau231) might contribute to the pathogenesis of AD.[32] The effect of 7β-OHC on tau phosphorylation was assessed in mouse hippocampal homogenates. 7β-OHC treatment caused an increase in p-tau262 levels but did not significantly affect p-tau199/202, p-tau 396, p-tau231, or total tau levels (Figure 11a, 11b). Ser262 is one of the major phosphorylation sites causing inhibition of the binding of Tau to microtubules.[105] These results indicate that 7β-OHC might affect tau phosphorylation at selective sites without affecting total tau levels.

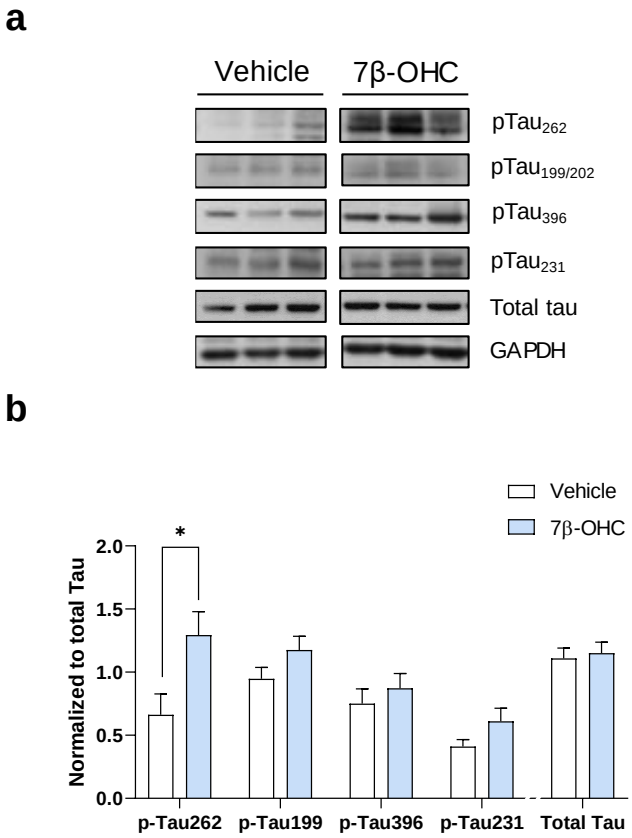

Figure 11. Effect of 7β-OHC on tau phosphorylation. Expression levels of total tau and phosphorylated tau (p-tau262, p-tau199, p-tau231, and p-tau396) were assayed in the homogenates of mouse hippocampus after 3 days of 7β-OHC treatment compared with those after vehicle treatment (n = 6 per group). The intensities of the target bands were quantified using Multigage software and normalized to total tau or GAPDH levels. *$p < 0.05$. Abbreviations: 7β-OHC, 7β-hydroxycholesterol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

7. 7β-OHC treatment induces perseveration

Touchscreen-based behavioral experiments were conducted to determine whether 7β-OHC treatment resulted in cognitive impairment. As both attention and response control abilities are impaired in the early stages of AD,[106,107] I used the 5-CSRT task to examine the behavioral effects of 7β-OHC. After a 1-week recovery from intracerebroventricular stereotaxic infusion, mice were used to perform baseline sessions of the 5-CSRT on 2 consecutive days with 2-second SD. In the baseline sessions, no differences in accuracy (Figure 12a) or omission (Figure 12b) were observed between mice injected with 7β-OHC and those with vehicle. Thus, these mice were moved onto probe sessions. The shortened SD stimuli were presented pseudo-randomly so that probe sessions receive more attention than the baseline sessions. There was no significant change in accuracy (Figure 13a) or omission (Figure 13g) between mice that received 7β-OHC injection and those that received the vehicle. Additionally, the level of impulsivity was not affected by 7β-OHC treatment (Figure 13d). However, mice that received 7β-OHC injection engaged in a significantly higher number of perseveration responses to incorrect stimuli (Figure 12b) but not to correct stimuli (Figure 13c). These deficits were not attributed to locomotor impairments following anesthesia and brain surgery since beam break rates (Figure 13e, 13f) and reward collection latencies were not altered by 7β-OHC treatment (Figure 13h, 13i).

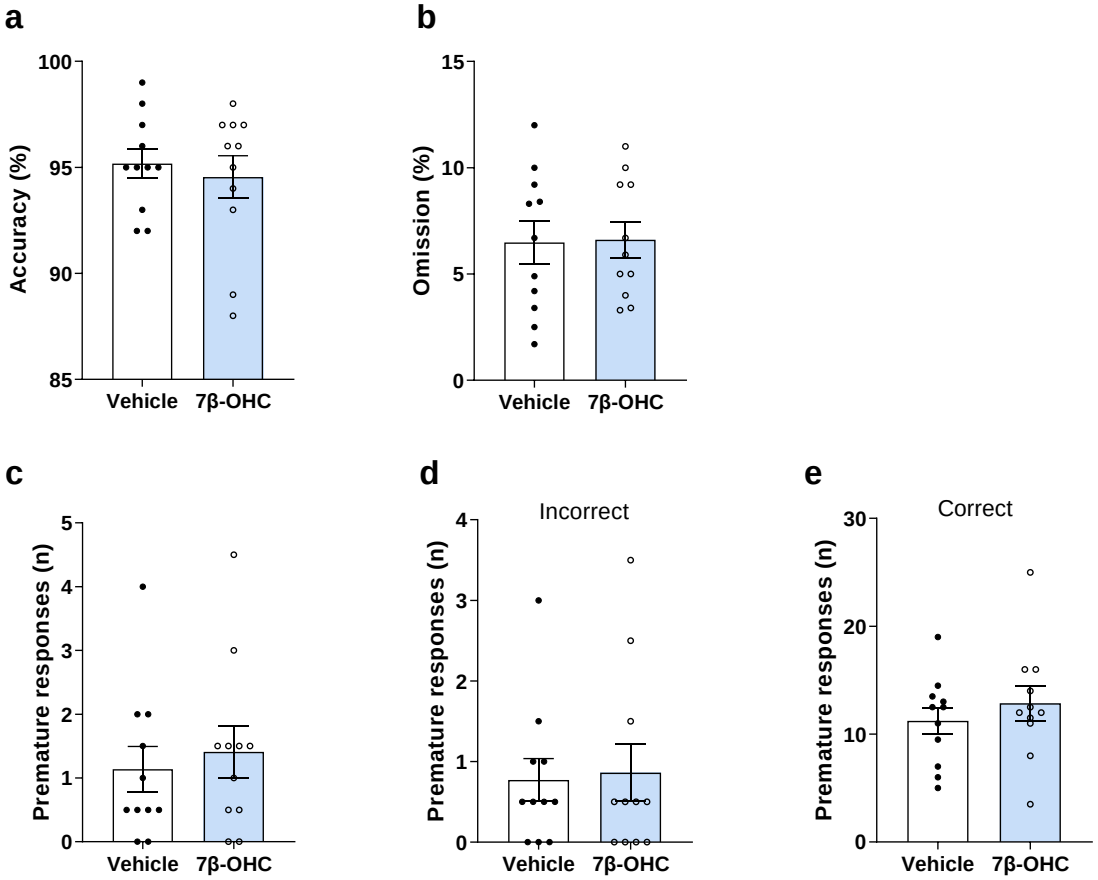

Figure 12. Baseline performance on the 5-CSRT task with 2.0-second SD. (a) Accuracy. (b) Omission. (c) Premature response. (d) Response to incorrect stimuli. (e) Response to correct stimuli. Data are presented as mean ± SEM. Abbreviations: 7β-OHC, 7β-hydroxycholesterol.

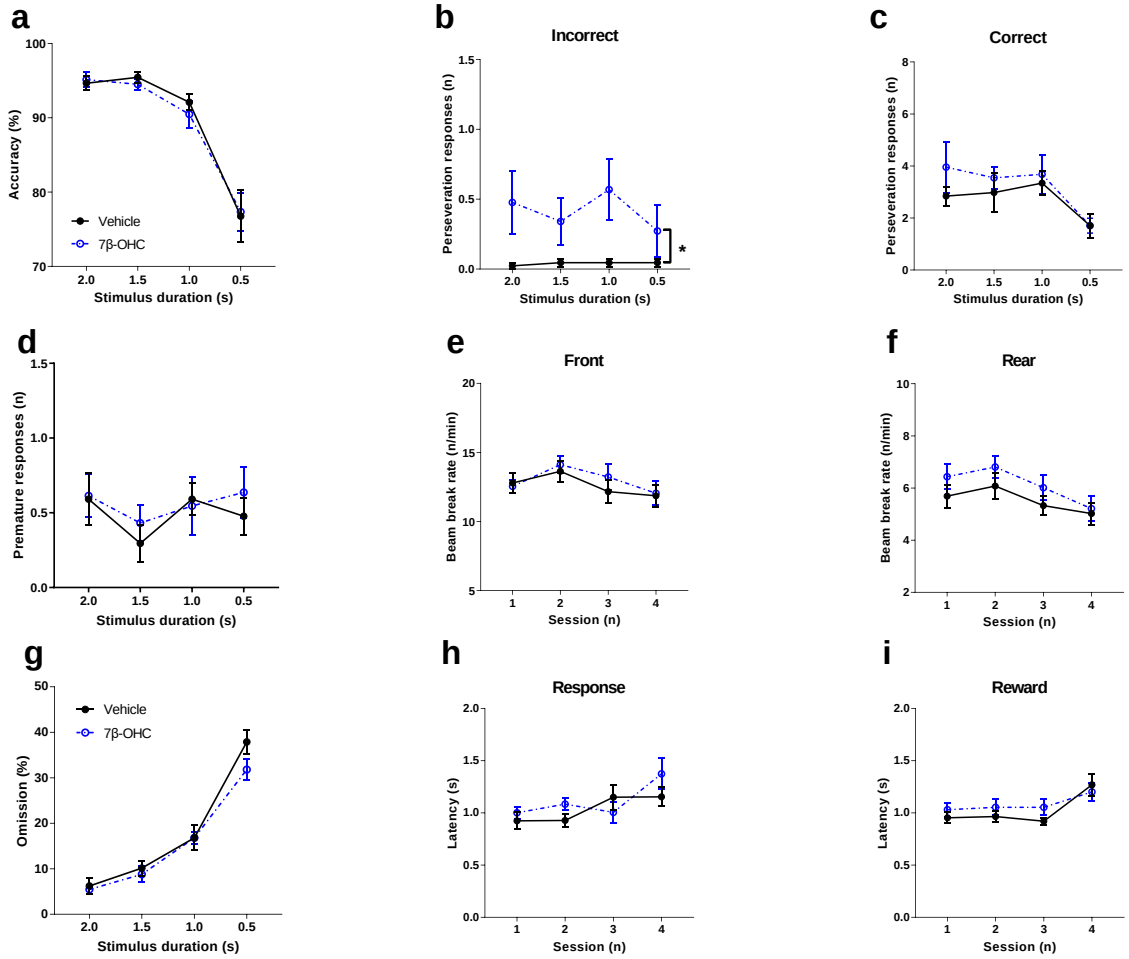

Figure 13. 7β-OHC injected mice showed perseveration in the 5-CSRT task. (a-f) 5-CSRT task performance. (a) Accuracy. Perseveration responses to (b) incorrect stimuli and (c) correct stimuli. (d) Premature responses. Beam break rate of (e) front and (f) rear sides of the chamber. n = 11 per group. (g-i) Levels of omission and motor activity during probe test. (g) Omission. (h) Response latency. (i) Reward latency. Data are presented as mean ± SEM. **p* < 0.05. Abbreviations: 7β-OHC, 7β-hydroxycholesterol.

IV. DISCUSSION

The aim of this study was to elucidate the role of 7β-OHC in AD pathology. Despite extensive research on AD pathogenesis and biomarkers, none of the previous efforts have been implemented in clinics. The present study showed that 7β-OHC levels in the human hair could be used as reliable and convenient peripheral biomarkers for AD-related cognitive decline, using the novel quantitative sterol signature derived by GC-MS. The animal studies showed that the treatment with 7β-OHC exerts potent effects on AD-related pathology. The results demonstrated that 7β-OHC induced significant neuroinflammation and promoted the activation of the amyloidogenic pathway while inhibiting the non-amyloidogenic pathway. Furthermore, p-tau262 levels were increased by 7β-OHC treatment. These results suggest that accumulation of 7β-OHC in the brain may play a role in AD pathogenesis.

1. Previous studies on 7β-OHC

Cholesterol is highly sensitive to auto-oxidation in free radical reactions. Free radical oxidation of cholesterol has been implicated in multiple diseases such as atherosclerosis, retinal degeneration, age-related macular degeneration, and Alzheimer's disease.[108-111] 7β-OHC is the major auto-oxidation product of cholesterol. Increased plasma 7β-OHC levels have been found in various metabolic disorders such as atherosclerosis, age-macular degeneration, and cardiovascular diseases.[108,112,113] High concentrations of 7β-OHC have been found in foam cells and fatty streaks, which are known hallmarks of atherosclerosis. However, most of the previous studies regarding 7β-OHC focused on cardiovascular diseases.

Increased levels of 7β-OHC have been found to induce cell death and inflammation.[114] Oxysterols such as 7β-OHC are known to exert pro-inflammatory effects though dynamic interaction with Liver X receptors (LXRs).[114,115] LXRs are oxysterol-activated nuclear receptors, and they have emerged as therapeutic targets for AD because the initiation and progression of the disease have been linked to

cholesterol metabolism.[116] LXRs are major components of intracellular cholesterol homeostasis, and oxysterols act as LXR ligands to regulate transcription associated with lipid metabolism.[117,118] LXRs control cellular cholesterol efflux by regulating the gene expression of cholesterol transport proteins, including the ATP-binding cassette (ABC) transporters ABCA1 and ABCG1, and ApoE.[119] In particular, ABCG1 is involved in the transport of 7β-OHC and plays a protective role against 7β-OHC-induced cell death.[120] ABCG1-deficient mice showed an increase in the number of apoptotic macrophages in vessel walls due to decreased export capacity of 7β-OHC.[121]

In addition, 7β-OHC is elevated in the rat hippocampus after excitotoxic neuronal injury induced by glutamate analogs.[122,123] APP and Aβ oxidizes cholesterol to form 7β-OHC.[90] However, the role of 7β-OHC in AD pathogenesis has not been fully determined. Previous studies have considered 7β-OHC one of the byproducts accompanying AD pathology, rather than a factor that significantly induces AD pathology.[90]

2. 7β-OHC induces the amyloidogenic pathway

In order to explore whether increased 7β-OHC is merely a byproduct of neurodegeneration or an active compound mediating AD pathology in the brain, I examined its role in APP metabolism. I found that stereotaxic 7β-OHC injection resulted in increased BACE1 expression and Aβ concentration in vivo. This finding contradicts that of a previous in vitro study in which 7β-OHC inhibited soluble APP secretion and TACE activity without affecting BACE1 levels.[90] 7β-OHC has also been reported to enhance the binding of Aβ in neuronal cell membranes by up-regulating the CD36/β1-integrin binding complex, which activates intracellular signal transduction cascades promoting pro-inflammatory responses.[69,124] The present study shows, for the first time, that 7β-OHC can increase BACE1 expression in vivo (Figure 10), and this is a critical finding as BACE1 is the rate-limiting enzyme for Aβ production. BACE1 expression is up-regulated in various conditions that cause cellular stress, including energy

deprivation, hypoxia, and oxidative stress.[125-127] 7β-OHC enhanced the amyloidogenic pathway, eventually increasing Aβ levels; however, TACE levels were significantly reduced after 7β-OHC treatment. Considering the relatively young age of the mice in this study, it is noteworthy to observe such an increase in Aβ after 3 days of 7β-OHC treatment. I found that the ability to suppress inaccurate stimuli, which is an indicator of frontal lobe function, was impaired in 7β-OHC treated mice (Figure 13b). The 7β-OHC-treated mice might be less sensitive to negative feedback (e.g., house light on and no reward in the magazine) compared to the vehicle-treated mice. The ventromedial frontal lobe is critical for learning from negative feedback.[128] Follow-up studies are required to determine the mechanisms that underlie such behavioral changes.

3. 7β-OHC induced neuroinflammation in relation to AD

Although inflammation plays an integral role in the progression of AD, the pro-inflammatory effect of 7β-OHC has not been fully investigated, particularly in the context of AD-related pathology. In neurodegenerative diseases such as AD, activated microglia and elevated inflammatory cytokine levels are observed around senile plaques, leading to APP stimulation and excess production of Aβ.[129,130] In turn, Aβ fibrils cause further microglial activation, resulting in a vicious cycle of neuronal loss and cognitive decline.[131] In this study, significant recruitment of Iba-1-positive microglia was observed in the brain of 7β-OHC-treated mice (Figure 6). Notably, 7β-OHC treatment induced secretion of pro-inflammatory cytokines including IL-1β and IL-6 (Figure 7). Both IL-1β and IL-6 levels were previously reported to be elevated in the brains of AD patients and APP transgenic mice.[87,132] The number of activated cortical microglia in AD patients has been significantly correlated with cognitive decline as reflected by MMSE scores, whereas amyloid deposition failed to demonstrate a significant clinical correlation.[133] In addition, significant cortical microglial activation has been observed in MCI patients independently of Aβ deposition,[134] and a recent study has shown that microglial activation, manifested by upregulated soluble triggering receptor expressed on myeloid cells (sTREM2), is already

present at the preclinical subjective cognitive decline (SCD) stage.[135] These findings suggest that neuroinflammation could be a phenomenon that occurs early in the disease process and persists as the disease progresses.[136,137]

4. Neuroprotective effects of statin may be related to 7β-OHC

Statins, cholesterol lowering drugs, have been reported to have neuroprotective effects in AD.[138] However, previous reports have shown inconsistent results, and the molecular mechanism of the effects of these drugs on AD is poorly understood. Additionally, the association of statins and AD is found to be different depending on the hydrophilic or lipophilic properties of statins.[139] It is interesting to note that the neuroprotective effect of statins against Aβ-induced toxicity is not correlated with changes in total cholesterol levels.[140] The levels of 7β-OHC in the plasma of middle-aged men with hypercholesterolemia were decreased after simvastatin treatment.[141] Since the mechanism of statin-induced cognitive protection in AD is still unclear, further studies are required to unravel the molecular underpinnings that link 7β-OHC and statins in AD. However, the results of our study may suggest that statins could be used for prevention or management of AD development. Given that 7β-OHC can exacerbate AD pathology, statins might alleviate AD pathology by reducing the levels of toxic cholesterol metabolites rather than total cholesterol. This might explain the inconsistent protective effect of statins on AD development, since all prior studies have measured the levels of total cholesterol, rather than those of its metabolites, which could vary from patient to patient. Therefore, future studies evaluating the effects of statins on AD pathology should assess the levels of different oxysterol derivatives such as 7β-OHC in participants.

5. Limitations

This study has several limitations. First, the sample size of the human participants was relatively small; thus, the results of this study should be interpreted with caution. Second, the effect of 7β-OHC

on oxidative stress was not investigated in this study. 7β-OHC has been reported to induce oxidative stress in 158 N murine oligodendrocytes.[142] Considering the fact that oxidative stress is one of the main etiologies of AD pathogenesis, oxidative stress induced by 7β-OHC may promote the amyloidogenic pathway, but additional studies are needed to further address this issue. Third, C57BL/6 mice, instead of APP/PS1 transgenic mice, were used in this study. Therefore, it was not possible to investigate how 7β-OHC exacerbates existing AD pathology. Further studies are needed to elucidate in detail the additional mechanisms underlying the role of 7β-OHC in AD. Despite its limitations, the study has several strengths. Since the cumulative levels of steroid molecules in hair are indicative of plasma levels over the past several months, this study suggests that increased plasma 7β-OHC is a preceding factor of AD and can increase AD pathology. These findings open an important avenue for the development of therapeutics for AD.

V. CONCLUSION

This study showed that increased levels of 7β-OHC in the human hair could be a reliable peripheral biomarker for AD. Further, the study demonstrated that 7β-OHC not only induces neuroinflammation but also affects APP processing by up-regulating BACE1 expression. Therefore, a therapeutic intervention that targets 7β-OHC may alleviate or prevent AD pathogenesis.

REFERENCES

1. Nam HJ, Hwang SH, Kim YJ, Kim KW. Korean dementia observatory 2018. Research Report. Sungnam; Ministry of Health and Welfare & National Institute of Dementia; 2018 December. Report No.: NIDR-1802-0023.

2. Hardy JA, Higgins GA. Alzheimer's disease: the amyloid cascade hypothesis. Science 1992;256:184-5.

3. Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 2002;297:353-6.

4. Nestor PJ, Scheltens P, Hodges JR. Advances in the early detection of Alzheimer's disease. Nat Med 2004;10 Suppl:S34-41.

5. Perrin RJ, Fagan AM, Holtzman DM. Multimodal techniques for diagnosis and prognosis of Alzheimer's disease. Nature 2009;461:916-22.

6. Jack CR, Jr., Knopman DS, Jagust WJ, Shaw LM, Aisen PS, Weiner MW, et al. Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade. Lancet Neurol 2010;9:119-28.

7. Herrup K, Carrillo MC, Schenk D, Cacace A, Desanti S, Fremeau R, et al. Beyond amyloid: getting real about non-amyloid targets in Alzheimer's disease. Alzheimers Dement 2013;9:452-8.e1.

8. Drachman DA. The amyloid hypothesis, time to move on: Amyloid is the downstream result, not cause, of Alzheimer's disease. Alzheimers Dement 2014;10:372-80.

9. Harrison JR, Owen MJ. Alzheimer's disease: the amyloid hypothesis on trial. Br J Psychiatry 2016;208:1-3.

10. Blass JP. Brain metabolism and brain disease: is metabolic deficiency the proximate cause of Alzheimer dementia? J Neurosci Res 2001;66:851-6.

11. Kivipelto M, Helkala EL, Laakso MP, Hanninen T, Hallikainen M, Alhainen K, et al. Apolipoprotein E epsilon4 allele, elevated midlife total cholesterol level, and high midlife systolic blood pressure are independent risk factors for late-life Alzheimer disease. Ann Intern Med 2002;137:149-55.

12. Anstey KJ, Lipnicki DM, Low LF. Cholesterol as a risk factor for dementia and cognitive decline: a systematic review of prospective studies with meta-analysis. Am J Geriatr Psychiatry 2008;16:343-54.

13. Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW, et al. Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. Science 1993;261:921-3.

14. Korade Z, Kenworthy AK. Lipid rafts, cholesterol, and the brain. Neuropharmacology 2008;55:1265-73.

15. Simons K, Ikonen E. How cells handle cholesterol. Science 2000;290:1721-6.

16. Dietschy JM, Turley SD. Thematic review series: brain Lipids. Cholesterol metabolism in the central nervous system during early development and in the mature animal. J Lipid Res 2004;45:1375-97.

17. Distl R, Meske V, Ohm TG. Tangle-bearing neurons contain more free cholesterol than adjacent tangle-free neurons. Acta Neuropathol 2001;101:547-54.

18. Puglielli L, Konopka G, Pack-Chung E, Ingano LA, Berezovska O, Hyman BT, et al. Acyl-coenzyme A: cholesterol acyltransferase modulates the generation of the amyloid beta-peptide. Nat Cell Biol 2001;3:905-12.

19. Runz H, Rietdorf J, Tomic I, de Bernard M, Beyreuther K, Pepperkok R, et al. Inhibition of intracellular cholesterol transport alters presenilin localization and amyloid precursor protein processing in neuronal cells. J Neurosci 2002;22:1679-89.

20. Maulik M, Westaway D, Jhamandas JH, Kar S. Role of cholesterol in APP metabolism and its significance in Alzheimer's disease pathogenesis. Mol Neurobiol 2013;47:37-63.

21. Shie F-S, Jin L-W, Cook DG, Leverenz JB, LeBoeuf RC. Diet-induced hypercholesterolemia enhances brain Aβ accumulation in transgenic mice. Neuroreport 2002;13:455-9.

22. Refolo LM, Malester B, LaFrancois J, Bryant-Thomas T, Wang R, Tint GS, et al. Hypercholesterolemia accelerates the Alzheimer's amyloid pathology in a transgenic mouse model. Neurobiol Dis 2000;7:321-31.

23. Wang R, Li Jing J, Diao S, Kwak Y-D, Liu L, Zhi L, et al. Metabolic stress modulates Alzheimer's β-secretase gene transcription via SIRT1-PPARγ-PGC-1 in neurons. Cell Metabolism 2013;17:685-94.

24. Pike LJ. Lipid rafts: bringing order to chaos. Journal of Lipid Research 2003;44:655-67.

25. Osenkowski P, Ye W, Wang R, Wolfe MS, Selkoe DJ. Direct and potent regulation of gamma-secretase by its lipid microenvironment. J Biol Chem 2008;283:22529-40.

26. Kalvodova L, Kahya N, Schwille P, Ehehalt R, Verkade P, Drechsel D, et al. Lipids as modulators of proteolytic activity of BACE: involvement of cholesterol, glycosphingolipids, and anionic phospholipids in vitro. J Biol Chem 2005;280:36815-23.

27. Vetrivel KS, Cheng H, Lin W, Sakurai T, Li T, Nukina N, et al. Association of gamma-secretase with lipid rafts in post-Golgi and endosome membranes. J Biol Chem 2004;279:44945-54.

28. Ehehalt R, Keller P, Haass C, Thiele C, Simons K. Amyloidogenic processing of the Alzheimer beta-amyloid precursor protein depends on lipid rafts. J Cell Biol 2003;160:113-23.

29. Harold D, Abraham R, Hollingworth P, Sims R, Gerrish A, Hamshere ML, et al. Genome-wide association study identifies variants at CLU and PICALM associated with Alzheimer's disease. Nat Genet 2009;41:1088-93.

30. Abuznait AH, Kaddoumi A. Role of ABC transporters in the pathogenesis of Alzheimer's disease. ACS Chem Neurosci 2012;3:820-31.

31. Young JE, Boulanger-Weill J, Williams DA, Woodruff G, Buen F, Revilla AC, et al. Elucidating molecular phenotypes caused by the SORL1 Alzheimer's disease genetic risk factor using human induced pluripotent stem cells. Cell Stem Cell 2015;16:373-85.

32. Johnson GV, Stoothoff WH. Tau phosphorylation in neuronal cell function and dysfunction. J Cell Sci 2004;117:5721-9.

33. Rahman A, Akterin S, Flores-Morales A, Crisby M, Kivipelto M, Schultzberg M, et al. High cholesterol diet induces tau hyperphosphorylation in apolipoprotein E deficient mice. FEBS Letters 2005;579:6411-6.

34. Fan QW, Yu W, Gong JS, Zou K, Sawamura N, Senda T, et al. Cholesterol-dependent modulation of dendrite outgrowth and microtubule stability in cultured neurons. J Neurochem 2002;80:178-90.

35. Wolozin B. Cholesterol and the biology of Alzheimer's disease. Neuron 2004;41:7-10.

36. Fantini J, Yahi N. Molecular insights into amyloid regulation by membrane cholesterol and sphingolipids: common mechanisms in neurodegenerative diseases. Expert Rev Mol Med 2010;12:e27.

37. Helzner EP, Luchsinger JA, Scarmeas N, Cosentino S, Brickman AM, Glymour MM, et al. Contribution of vascular risk factors to the progression in Alzheimer disease. Arch Neurol 2009;66:343-8.

38. Kivipelto M, Helkala EL, Laakso MP, Hanninen T, Hallikainen M, Alhainen K, et al. Midlife vascular risk factors and Alzheimer's disease in later life: longitudinal, population based study. BMJ 2001;322:1447-51.

39. Solomon A, Kivipelto M, Wolozin B, Zhou J, Whitmer RA. Midlife serum cholesterol and increased risk of Alzheimer's and vascular dementia three decades later. Dement Geriatr Cogn Disord 2009;28:75-80.

40. Anstey KJ, Ashby-Mitchell K, Peters R. Updating the evidence on the association between serum cholesterol and risk of late-life dementia: Review and meta-analysis. J Alzheimers Dis 2017;56:215-28.

41. Tan ZS, Seshadri S, Beiser A, Wilson PW, Kiel DP, Tocco M, et al. Plasma total cholesterol level as a risk factor for Alzheimer disease: the Framingham Study. Arch Intern Med 2003;163:1053-7.

42. Reitz C, Tang M-X, Manly J, Schupf N, Mayeux R, Luchsinger JA. Plasma lipid levels in the elderly are not associated with the risk of mild cognitive impairment. Dement Geriatr Cogn Disord 2008;25:232-7.

43. Burns M, Gaynor K, Olm V, Mercken M, LaFrancois J, Wang L, et al. Presenilin redistribution associated with aberrant cholesterol transport enhances beta-amyloid production in vivo. J Neurosci 2003;23:5645-9.

44. Ogasawara F, Kano F, Murata M, Kimura Y, Kioka N, Ueda K. Changes in the asymmetric distribution of cholesterol in the plasma membrane influence streptolysin O pore formation. Sci Rep 2019;9:4548.

45. Martins IJ, Hone E, Foster JK, Sünram-Lea SI, Gnjec A, Fuller SJ, et al. Apolipoprotein E, cholesterol metabolism, diabetes, and the convergence of risk factors for Alzheimer's disease

and cardiovascular disease. Mol Psychiatr 2006;11:721-36.

46. Puglielli L, Tanzi RE, Kovacs DM. Alzheimer's disease: the cholesterol connection. Nat Neurosci 2003;6:345-51.

47. Casserly I, Topol E. Convergence of atherosclerosis and Alzheimer's disease: inflammation, cholesterol, and misfolded proteins. Lancet 2004;363:1139-46.

48. Farroqui AA. Lipid mediators and their metabolism in the brain Springer; 2011. p.267-97.

49. Ghribi O. Potential mechanisms linking cholesterol to Alzheimer's disease-like pathology in rabbit brain, hippocampal organotypic slices, and skeletal muscle. J Alzheimers Dis 2008;15:673-84.

50. Mateos L, Akterin S, Gil-Bea F-J, Spulber S, Rahman A, Björkhem I, et al. Activity-Regulated cytoskeleton-associated protein in rodent brain is down-regulated by high fat diet in vivo and by 27-hydroxycholesterol in vitro. Brain Pathol (Zurich, Switzerland) 2008;19:69-80.

51. Park SH, Kim JH, Choi KH, Jang YJ, Bae SS, Choi BT, et al. Hypercholesterolemia accelerates amyloid β-induced cognitive deficits. Int J Mol Med 2013;31:577-82.

52. Gamba P, Testa G, Gargiulo S, Staurenghi E, Poli G, Leonarduzzi G. Oxidized cholesterol as the driving force behind the development of Alzheimer's disease. Front Aging Neurosci 2015;7:119.

53. Guardiola F, Codony R, Addis PB, Rafecas M, Boatella J. Biological effects of oxysterols: current status. Food Chem Toxicol 1996;34:193-211.

54. Russell DW. Oxysterol biosynthetic enzymes. Biochim Biophys Acta 2000;1529:126-35.

55. Russell DW, Halford RW, Ramirez DM, Shah R, Kotti T. Cholesterol 24-hydroxylase: an enzyme of cholesterol turnover in the brain. Annu Rev Biochem 2009;78:1017-40.

56. Bjorkhem I. Crossing the barrier: oxysterols as cholesterol transporters and metabolic modulators in the brain. J Intern Med 2006;260:493-508.

57. Bjorkhem I, Cedazo-Minguez A, Leoni V, Meaney S. Oxysterols and neurodegenerative diseases. Mol Aspects Med 2009;30:171-9.

58. Heverin M, Meaney S, Lutjohann D, Diczfalusy U, Wahren J, Bjorkhem I. Crossing the barrier: net flux of 27-hydroxycholesterol into the human brain. J Lipid Res 2005;46:1047-52.

59. Lutjohann D, Breuer O, Ahlborg G, Nennesmo I, Siden A, Diczfalusy U, et al. Cholesterol homeostasis in human brain: evidence for an age-dependent flux of 24S-hydroxycholesterol from the brain into the circulation. Proc Natl Acad Sci U S A 1996;93:9799-804.

60. Olkkonen VM, Béaslas O, Nissilä E. Oxysterols and their cellular effectors. Biomolecules; 2012;2:76-103.

61. Mielke MM, Zandi PP, Sjogren M, Gustafson D, Ostling S, Steen B, et al. High total cholesterol levels in late life associated with a reduced risk of dementia. Neurology 2005;64:1689-95.

62. Popp J, Meichsner S, Kolsch H, Lewczuk P, Maier W, Kornhuber J, et al. Cerebral and extracerebral cholesterol metabolism and CSF markers of Alzheimer's disease. Biochem Pharmacol 2013;86:37-42.

63. Moutinho M, Nunes MJ, Rodrigues E. Cholesterol 24-hydroxylase: Brain cholesterol metabolism and beyond. Biochim Biophys Acta 2016;1861:1911-20.

64. Hughes TM, Rosano C, Evans RW, Kuller LH. Brain cholesterol metabolism, oxysterols, and dementia. J Alzheimers Dis 2013;33:891-911.

65. Shafaati M, Marutle A, Pettersson H, Lövgren-Sandblom A, Olin M, Pikuleva I, et al. Marked accumulation of 27-hydroxycholesterol in the brains of Alzheimer's patients with the Swedish

APP 670/671 mutation. J Lipid Res 2011;52:1004-10.

66. Marwarha G, Dasari B, Prasanthi JRP, Schommer J, Ghribi O. Leptin reduces the accumulation of Abeta and phosphorylated tau induced by 27-hydroxycholesterol in rabbit organotypic slices. J Alzheimers Dis 2010;19:1007-19.

67. Prasanthi JR, Huls A, Thomasson S, Thompson A, Schommer E, Ghribi O. Differential effects of 24-hydroxycholesterol and 27-hydroxycholesterol on beta-amyloid precursor protein levels and processing in human neuroblastoma SH-SY5Y cells. Mol Neurodegener 2009;4:1.

68. Heverin M, Maioli S, Pham T, Mateos L, Camporesi E, Ali Z, et al. 27-Hydroxycholesterol mediates negative effects of dietary cholesterol on cognition in mice. Behav Brain Res 2015;278:356-9.

69. Gamba P, Leonarduzzi G, Tamagno E, Guglielmotto M, Testa G, Sottero B, et al. Interaction between 24-hydroxycholesterol, oxidative stress, and amyloid-beta in amplifying neuronal damage in Alzheimer's disease: three partners in crime. Aging Cell 2011;10:403-17.

70. Gamba P, Staurenghi E, Testa G, Giannelli S, Sottero B, Leonarduzzi G. A crosstalk between brain cholesterol oxidation and glucose metabolism in Alzheimer’s disease. Front Neurosci 2019;13.

71. Zmijewski M, Skobowiat C, Zbytek B, Slominski R, Steketee J. Sensing the environment: Regulation of local and global homeostasis by the skin neuroendocrine system. Adv Anatomy Embryol Cell Biol 2012;212:v, vii, 1-115.

72. Russell E, Koren G, Rieder M, Van Uum S. Hair cortisol as a biological marker of chronic stress: current status, future directions and unanswered questions. Psychoneuroendocrinology 2012;37:589-601.

73. Choi MH, Kim KR, Kim IS, Lho DS, Chung BC. Increased hair polyamine levels in patients

with Alzheimer's disease. Ann Neurol 2001;50:128.

74. Do Yup Lee EK, Choi MH. Technical and clinical aspects of cortisol as a biochemical marker of chronic stress. BMB Rep 2015;48:209.

75. Cho SH, Choi MH, Sim WY, Lee WY, Chung BC. Metabolic alterations of DHEA and cholesterol sulphates in the hair of patients with acne measured by liquid chromatography-mass spectrometry. Exp Dermatol 2010;19:694-6.

76. Veldhorst MA, Noppe G, Jongejan MH, Kok CB, Mekic S, Koper JW, et al. Increased scalp hair cortisol concentrations in obese children. J Clin Endocrinol Metab 2014;99:285-90.

77. Hegsted D, Nicolosi RJ. Individual variation in serum cholesterol levels. Proc Natl Acad Sci 1987;84:6259-61.

78. Son HH, Lee DY, Seo HS, Jeong J, Moon JY, Lee JE, et al. Hair sterol signatures coupled to multivariate data analysis reveal an increased 7beta-hydroxycholesterol production in cognitive impairment. J Steroid Biochem Mol Biol 2016;155:9-17.

79. Jung HJ, Kim SJ, Lee WY, Chung BC, Choi MH. Gas chromatography/mass spectrometry based hair steroid profiling may reveal pathogenesis in hair follicles of the scalp. Rapid Commun Mass Spectrom 2011;25:1184-92.

80. Poli G, Sottero B, Gargiulo S, Leonarduzzi G. Cholesterol oxidation products in the vascular remodeling due to atherosclerosis. Mol Aspects Med 2009;30:180-9.

81. Prunet C, Montange T, Vejux A, Laubriet A, Rohmer JF, Riedinger JM, et al. Multiplexed flow cytometric analyses of pro- and anti-inflammatory cytokines in the culture media of oxysterol-treated human monocytic cells and in the sera of atherosclerotic patients. Cytometry A 2006;69:359-73.

82. Ares MP, Porn-Ares MI, Moses S, Thyberg J, Juntti-Berggren L, Berggren P, et al. 7beta-

hydroxycholesterol induces Ca(2+) oscillations, MAP kinase activation and apoptosis in human aortic smooth muscle cells. Atherosclerosis 2000;153:23-35.

83. Schroepfer GJ, Jr. Oxysterols: modulators of cholesterol metabolism and other processes. Physiol Rev 2000;80:361-554.

84. Roussi S, Gosse F, Aoude-Werner D, Zhang X, Marchioni E, Geoffroy P, et al. Mitochondrial perturbation, oxidative stress and lysosomal destabilization are involved in 7beta-hydroxysitosterol and 7beta-hydroxycholesterol triggered apoptosis in human colon cancer cells. Apoptosis 2007;12:87-96.

85. Hascalovici JR, Vaya J, Khatib S, Holcroft CA, Zukor H, Song W, et al. Brain sterol dysregulation in sporadic AD and MCI: relationship to heme oxygenase-1. J Neurochem 2009;110:1241-53.

86. Guardiola F. Cholesterol and phytosterol oxidation products: analysis, occurrence, and biological effects: AOCS Publishing; 2002.

87. Testa G, Staurenghi E, Zerbinati C, Gargiulo S, Iuliano L, Giaccone G, et al. Changes in brain oxysterols at different stages of Alzheimer's disease: Their involvement in neuroinflammation. Redox Biol 2016;10:24-33.

88. Leoni V, Lutjohann D, Masterman T. Levels of 7-oxocholesterol in cerebrospinal fluid are more than one thousand times lower than reported in multiple sclerosis. J Lipid Res 2005;46:191-5.

89. Hughes H, Mathews B, Lenz ML, Guyton JR. Cytotoxicity of oxidized LDL to porcine aortic smooth muscle cells is associated with the oxysterols 7-ketocholesterol and 7-hydroxycholesterol. Arterioscler Thromb 1994;14:1177-85.

90. Nelson TJ, Alkon DL. Oxidation of cholesterol by amyloid precursor protein and beta-amyloid

peptide. J Biol Chem 2005;280:7377-87.

91. Moon J-Y, Choi MH, Kim J. Metabolic profiling of cholesterol and sex steroid hormones to monitor urological diseases. Endocr Relat Cancer 2016;23:R455-R67.

92. American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders: DSM-IV-TR. Washington, DC: American Psychiatric Association, 2000.

93. Petersen RC. Mild cognitive impairment as a diagnostic entity. J Intern Med 2004;256:183-94.

94. Paxinos G, and Franklin, K. B. J. The mouse brain in stereotaxic coordinates (Deluxe Edition). 2nd ed. San Diego, CA: Academic Press; 2001.

95. White MA, Kim E, Duffy A, Adalbert R, Phillips BU, Peters OM, et al. TDP-43 gains function due to perturbed autoregulation in a Tardbp knock-in mouse model of ALS-FTD. Nat Neurosci 2018;21:552.

96. Mar AC, Horner AE, Nilsson SRO, Alsiö J, Kent BA, Kim CH, et al. The touchscreen operant platform for assessing executive function in rats and mice. Nat Protoc 2013;8:1985-2005.

97. Zeleznikow-Johnston AM, Renoir T, Churilov L, Li S, Burrows EL, Hannan AJ. Touchscreen testing reveals clinically relevant cognitive abnormalities in a mouse model of schizophrenia lacking metabotropic glutamate receptor 5. Sci Rep 2018;8:16412.

98. Cardinal RN, Aitken MR. Whisker: a client—server high-performance multimedia research control system. Behav Res Methods 2010;42:1059-71.

99. Robbins T. The 5-choice serial reaction time task: Behavioural pharmacology and functional neurochemistry. Psychopharmacology 2002;163:362-80.

100. Thelen KM, Lütjohann D, Vesalainen R, Janatuinen T, Knuuti J, von Bergmann K, et al. Effect

of pravastatin on plasma sterols and oxysterols in men. Eur J Clin Pharmacol 2006;62:9-14.

101. Hickman SE, Allison EK, El Khoury J. Microglial dysfunction and defective beta-amyloid clearance pathways in aging Alzheimer's disease mice. J Neurosci 2008;28:8354-60.

102. Wang WY, Tan MS, Yu JT, Tan L. Role of pro-inflammatory cytokines released from microglia in Alzheimer's disease. Ann Translat Med 2015;3:136-.

103. Rock KL, Kono H. The inflammatory response to cell death. Annu Rev Pathol 2008;3:99-126.

104. O'Callaghan YC, Woods JA, O'Brien NM. Characteristics of 7 beta-hydroxycholesterol-induced cell death in a human monocytic blood cell line, U937, and a human hepatoma cell line, HepG2. Toxicol In Vitro 2002;16:245-51.

105. Sengupta A, Kabat J, Novak M, Wu Q, Grundke-Iqbal I, Iqbal K. Phosphorylation of tau at both Thr 231 and Ser 262 is required for maximal inhibition of its binding to microtubules. Arch Biochem Biophys 1998;357:299-309.

106. Perry RJ, Hodges JR. Attention and executive deficits in Alzheimer's disease: A critical review. Brain 1999;122:383-404.

107. Perry RJ, Watson P, Hodges JR. The nature and staging of attention dysfunction in early (minimal and mild) Alzheimer’s disease: relationship to episodic and semantic memory impairment. Neuropsychologia 2000;38:252-71.

108. Brown AJ, Jessup W. Oxysterols and atherosclerosis. Atherosclerosis 1999;142:1-28.

109. Rodriguez IR, Larrayoz IM. Cholesterol oxidation in the retina: implications of 7KCh formation in chronic inflammation and age-related macular degeneration. J Lipid Res 2010;51:2847-62.

110. Rodriguez IR, Fliesler SJ. Photodamage generates 7-keto- and 7-hydroxycholesterol in the rat

retina via a free radical-mediated mechanism. Photochem Photobiol 2009;85:1116-25.

111. Vaya J, Schipper HM. Oxysterols, cholesterol homeostasis, and Alzheimer disease. J Neurochem 2007;102:1727-37.

112. Colles SM, Maxson JM, Carlson SG, Chisolm GM. Oxidized LDL-induced injury and apoptosis in atherosclerosis: potential roles for oxysterols. Trends Cardiovasc Med 2001;11:131-8.

113. Malvitte L, Montange T, Joffre C, Vejux A, Maïza C, Bron A, et al. Analogies entre processus athéromateux et dégénérescence maculaire liée à l'âge: rôles présumés des oxystérols. Journal Français d'Ophtalmologie 2006;29:570-8.

114. Vejux A, Malvitte L, Lizard G. Side effects of oxysterols: cytotoxicity, oxidation, inflammation, and phospholipidosis. Braz J Med Biol Res 2008;41:545-56.

115. Jusakul A, Yongvanit P, Loilome W, Namwat N, Kuver R. Mechanisms of oxysterol-induced carcinogenesis. Lipids Health Dis 2011;10:44.

116. Sodhi RK, Singh N. Liver X receptors: Emerging therapeutic targets for Alzheimer's disease. Pharmacol Res 2013;72:45-51.

117. Venkateswaran A, Laffitte BA, Joseph SB, Mak PA, Wilpitz DC, Edwards PA, et al. Control of cellular cholesterol efflux by the nuclear oxysterol receptor LXR alpha. Proc Natl Acad Sci U S A 2000;97:12097-102.

118. Janowski BA, Willy PJ, Devi TR, Falck JR, Mangelsdorf DJ. An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. Nature 1996;383:728-31.

119. Calkin AC, Tontonoz P. Transcriptional integration of metabolism by the nuclear sterol-activated receptors LXR and FXR. Nat Rev Mol Cell Biol 2012;13:213-24.

120. Engel T, Kannenberg F, Fobker M, Nofer JR, Bode G, Lueken A, et al. Expression of ATP binding cassette-transporter ABCG1 prevents cell death by transporting cytotoxic 7beta-hydroxycholesterol. FEBS Lett 2007;581:1673-80.

121. Baldán Á, Pei L, Lee R, Tarr P, Tangirala RK, Weinstein MM, et al. Impaired development of atherosclerosis in hyperlipidemic ldlr-/- and apoE-/- mice transplanted with abcg1-/- bone marrow. Arterioscler Thromb Vasc Biol 2006;26:2301-7.

122. Kim JH, Jittiwat J, Ong WY, Farooqui AA, Jenner AM. Changes in cholesterol biosynthetic and transport pathways after excitotoxicity. J Neurochem 2010;112:34-41.

123. Kim JH, Ee SM, Jittiwat J, Ong ES, Farooqui A, Jenner A, et al. Increased expression of acyl-coenzyme A: cholesterol acyltransferase-1 and elevated cholesteryl esters in the hippocampus after excitotoxic injury. Neuroscience 2011;185:125-34.

124. Bamberger ME, Harris ME, McDonald DR, Husemann J, Landreth GE. A cell surface receptor complex for fibrillar beta-amyloid mediates microglial activation. J Neurosci 2003;23:2665-74.

125. O'Connor T, Sadleir KR, Maus E, Velliquette RA, Zhao J, Cole SL, et al. Phosphorylation of the translation initiation factor eIF2alpha increases BACE1 levels and promotes amyloidogenesis. Neuron 2008;60:988-1009.

126. Guglielmotto M, Aragno M, Autelli R, Giliberto L, Novo E, Colombatto S, et al. The up-regulation of BACE1 mediated by hypoxia and ischemic injury: role of oxidative stress and HIF1alpha. J Neurochem 2009;108:1045-56.

127. Jo DG, Arumugam TV, Woo HN, Park JS, Tang SC, Mughal M, et al. Evidence that gamma-secretase mediates oxidative stress-induced beta-secretase expression in Alzheimer's disease. Neurobiol Aging 2010;31:917-25.

128. Wheeler EZ, Fellows LK. The human ventromedial frontal lobe is critical for learning from

negative feedback. Brain 2008;131:1323-31.

129. Wang WY, Tan MS, Yu JT, Tan L. Role of pro-inflammatory cytokines released from microglia in Alzheimer's disease. Ann Transl Med 2015;3:136.

130. Yates SL, Burgess LH, Kocsis-Angle J, Antal JM, Dority MD, Embury PB, et al. Amyloid beta and amylin fibrils induce increases in proinflammatory cytokine and chemokine production by THP-1 cells and murine microglia. J Neurochem 2000;74:1017-25.

131. Patel NS, Paris D, Mathura V, Quadros AN, Crawford FC, Mullan MJ. Inflammatory cytokine levels correlate with amyloid load in transgenic mouse models of Alzheimer's disease. J Neuroinflammation 2005;2:9.

132. Testa G, Staurenghi E, Zerbinati C, Gargiulo S, Iuliano L, Giaccone G, et al. Changes in brain oxysterols at different stages of Alzheimer's disease: Their involvement in neuroinflammation. Redox Biol 2016;10:24-33.

133. Edison P, Archer HA, Gerhard A, Hinz R, Pavese N, Turkheimer FE, et al. Microglia, amyloid, and cognition in Alzheimer's disease: An [11C](R)PK11195-PET and [11C]PIB-PET study. Neurobiol Dis 2008;32:412-9.

134. Okello A, Edison P, Archer HA, Turkheimer F, Kennedy J, Bullock R, et al. Microglial activation and amyloid deposition in mild cognitive impairment: A PET study. Neurology 2009;72:56-62.

135. Nordengen K, Kirsebom B-E, Henjum K, Selnes P, Gísladóttir B, Wettergreen M, et al. Glial activation and inflammation along the Alzheimer’s disease continuum. J Neuroinflamm 2019;16:46.

136. Heneka MT, Carson MJ, El Khoury J, Landreth GE, Brosseron F, Feinstein DL, et al. Neuroinflammation in Alzheimer's disease. Lancet Neurol 2015;14:388-405.

137. Edison P, Ahmed I, Fan Z, Hinz R, Gelosa G, Ray Chaudhuri K, et al. Microglia, amyloid, and glucose metabolism in Parkinson's disease with and without dementia. Neuropsychopharmacology 2013;38:938-49.

138. van der Most PJ, Dolga AM, Nijholt IM, Luiten PG, Eisel UL. Statins: mechanisms of neuroprotection. Prog Neurobiol 2009;88:64-75.

139. Sinyavskaya L, Gauthier S, Renoux C, Dell'Aniello S, Suissa S, Brassard P. Comparative effect of statins on the risk of incident Alzheimer disease. Neurology 2018;90: e179-e87.

140. Fonseca AC, Proenca T, Resende R, Oliveira CR, Pereira CM. Neuroprotective effects of statins in an in vitro model of Alzheimer's disease. J Alzheimers Dis 2009;17:503-17.

141. Dias IHK, Milic I, Lip GYH, Devitt A, Polidori MC, Griffiths HR. Simvastatin reduces circulating oxysterol levels in men with hypercholesterolaemia. Redox Biol 2018;16:139-45.

142. Sghaier R, Nury T, Leoni V, Caccia C, Pais De Barros JP, Cherif A, et al. Dimethyl fumarate and monomethyl fumarate attenuate oxidative stress and mitochondrial alterations leading to oxiapoptophagy in 158N murine oligodendrocytes treated with 7β-hydroxycholesterol. J Steroid Biochem Mol Biol 2019;194:105432.

ABSTRACT (IN KOREAN)

7β-Hydroxycholesterol 이 알츠하이머 치매 병인 기전에 미치는 영향과 말초 바이오마커로써의 활용 가능성 탐색

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하정희

콜레스테롤 대사 이상과 알츠하이머 병인 기전과의 관련성이 지속적으로 보고됨에 따라, 콜레스테롤 대사체인 산화 콜레스테롤에 대한 연구가 진행되어왔으나, 산화 콜레스테롤이 치매 병리에 미치는 영향에 대해서는 잘 알려져 있지 않다. 특히 동맥경화와 관련된 것으로 알려진 7beta-hydroxycholesterol(7β-OHC)와 치매 병리와의 관련성은 충분히 연구되지 않았다. 치매 환자의 혈액, 뇌척수액에서 7β-OHC이 증가되어 있다는 것이 꾸준히 보고되어 왔지만, 이것이 알츠하이머병이 진행됨에 따라 증가되는 부산물인지, 핵심 치매 병리와 관련된 요인 인지에 대해서는 잘 알려진 바가 없다. 한편, 모발내 누적된 콜레스테롤 수준은 지난 몇 개월 동안의 혈장 콜레스테롤 노출 수준을 반영한다고 알려져 있으며, 상대적으로 외부요인에 의한 영향이 적어 안정적인 콜레스테롤 측정을 가능케하는 생체 산물이다. 이에 본 연구에서 혈액이 아닌 모발에서 7β-OHC가 알츠하이머 말초 바이오마커로 활용될 수 있는지에 대한 가능성을 환자 군에서 탐색하고, 동물실험을 통해 7β-OHC이 치매 병리에 미치는 효과를 직접적으로 관찰하고자 하였다. 치매 진단에 따라 참여자를 세 군(정상인지, 경도인지

장애 치매)으로 나눈 뒤, 기체 크로마토그래피 질량 분석법(gas chromatography-mass spectrometry, GC-MS)을 통해 모발에서의 7β-OHC 수준을 측정하였고, 이후 인지 기능과 관련된 임상 지표와의 관련성을 분석하였다. 실제 뇌 안에서 7β-OHC의 역할을 조사하기 위해, 마우스 양쪽 해마에 7β-OHC를 주입한 뒤 대표적인 알츠하이머 병인 기전인 아밀로이드 베타, 타우 인산화 및 신경염증의 변화를 관찰하였다. 또한, 7β-OHC의 주입에 따른 인지 기능에 대한 영향을 평가하기 위해 터치스크린을 기반으로 한 행동 실험을 진행하였다. 분석 결과, 치매 환자 군과 경도인지장애의 모발에서 정상 군에 비해 7β-OHC 레벨이 유의미하게 증가하였으며, 연령, 성별, 교육 수준 및 스타틴(statin) 사용여부를 보정한 후에도 7β-OHC 과 간이정신상태검사 및 전반적 퇴화척도와 유의미한 상관관계가 관찰되었다. 마우스 실험에서 7β-OHC를 주입한 군에서 미세아교세포와 별아교세포의 활성화가 관찰되었으며, 대표적인 염증 사이토카인인 IL-1β 와 IL-6 분비가 증가하였다. 특징적으로 7β-OHC를 주입 후 BACE1 단백질의 발현이 유의미하게 증가되고, 반대로 TACE 레벨이 감소하는 등 전반적인 amyloidogenic pathway 가 증가되었다. 추가적인 행동 실험에서, 7β-OHC를 주입한 군에서 전두엽 기능장애를 시사하는 보속증이 관찰되었다. 결과적으로 본 연구는 모발에서 채취한 7β-OHC의 변화가 인지 저하 및 치매 위험성과 관련성이 있음을 증명하였으며, 7β-OHC 주입이 직접적으로 치매 병리에 영향을 주는 것을 확인하였다. 모발에서 콜레스테롤 축적량이 일정 기간 동안의 혈장 노출 수준을 나타낸다는 점을 고려하면, 본 연구는 증가된 혈장 7β-OHC이 알츠하이머 병리를 증가시킬 수 있는 선행 인자 일 수 있음을 시사한다.

핵심 되는 말: 옥시스테롤, 7β-하이드록시콜레스테롤, 헤어 콜레스테롤, 신경 염증, 알츠하이머 치매