Comparison of protective effects of four polyphenols on neuropathology and behavior of APP/PS1-21 transgenic mice, a model of Alzheimer's disease

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Chaoyun Li

aus

China

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Dekan: Professor Dr. I. B. Autenrieth

1. Berichterstatter: Professor Dr. Dr. H. Schlüsener

2. Berichterstatter: Professor Dr. O. Kohlbacher

Dedicate To My Beloved Family

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Abbreviations

Aβ	Amyloid beta
ABCA1	ATP-binding cassette transporter A1
AD	Alzheimer's disease
AChE	Acetylcholinesterase
ADH	Alcohol dehydrogenase
ALDH	Acetaldehyde dehydrogenase
ANOVA	Analysis of Variance
AP-1	Activator protein-1
APP	Amyloid precursor protein
Akt	Serine/threonine kinase
BACE1	Beta-site APP cleaving enzyme-1
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
cGMP	Cyclic guanosine monophosphate
CASP	Caspase
CBF	Cerebrospinal fluid
COX	Cyclooxygenase
CMC	Carboxymethylcellulose
DHM	Dihydromyricetin
ERK	Extracellular signal-regulated kinases
FAD	Familial Alzheimer's disease
HIT	Herbal Ingredients' Targets database
IL	Interleukin
I-κB	Inhibitor-κB
GABA	Neurotransmitter gamma-aminobutyric acid
GSH	Glutathione synthetase
GSK-3β	Glycogen synthase kinase-3 beta
HMOX1	Heme oxygenase 1

HSP70	Heat Shock Protein 70
IHC	Immunohistochemistry
iNOS	Inducible NO synthase
JNK	c-Jun N-terminal kinase
KEGG	Kyoto Encyclopedia of Genes and Genomes
LOAD	Late onset Alzheimer's disease
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
NF-κB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 3
NFTs	Neurofibrillary tangles
NO	Nitric oxide
NRF2	Nuclear factor erythroid 2-related factor 2
PDE5	Phoshphodiesterase-5
PI3K	Phosphatidylinositol 3-kinase regulatory subunit alpha
PPAR	Peroxisome proliferator-activated receptor
PS	Presenilin
PKB	RAC-alpha serine/threonine-protein kinase
PKC	Protein kinase C
SAD	Sporadic Alzheimer's disease
SEM	Standard errors of means
STAT3	Signal transducer and activator of transcription 3
TCM	Traditional Chinese Medicine
TGF-β	Transforming growth factor-β
TTD	Therapeutic Target Database
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-α
VEGF-A	Vascular endothelial growth factor A

Chapter I: Introduction

In the year of 1901, the German neuropathologist and psychiatrist Dr. Alois Alzheimer made his first examination of a 51-years old woman named Auguste Deter who was suffering from progressive dementia, characterized by cognitive dysfunction and memory loss. Auguste Deter died in 1906. Her brain was analyzed and for the first time, Alois Alzheimer described extracellular miliary foci and intracellular dense bundle of fibrils as pathological hallmarks of this disease. In this year, he gave a lecture at a psychiatry congress in Tuebingen, Germany, about his new findings and published the results one year after. In 1910, Emil Kraepelin honored the study of Alois Alzheimer by naming the disease after him. Alois Alzheimer's observations, which are now regarded as neurofibrillary tangles (NFTs) and senile plaques, are still diagnostic features of Alzheimer's disease (AD) post mortem.

AD is a progressive neurodegenerative illness, and is now the most common form of dementia among the aging population, accounting for more than half of cases in clinical series and at autopsy (Bertram and Tanzi, 2012; Querfurth and LaFerla, 2010). Confusion, mood swings and insomnia are the initial symptoms of AD. Along with impaired memory, patients suffer from the devastating illness and lose the ability to perform activities of daily living. Finally, these patients are unable to perform even the simplest tasks and the cause of death is often a normally harmless infection in the very late phase of the disease (Forstl and Kurz, 1999).

1.1 Pathological features of Alzheimer's disease

AD is a neurodegenerative disease clinically characterized by progressive cognitive deterioration, neuropsychiatric and behavioral symptoms. Neuropathological examination of the brains of AD patients reveals extracellular deposits of amyloid beta (Aβ) in brain parenchyma and increased neuro-inflammation (Mattson, 2004; Reddy et al., 2010; Selkoe, 2001)

1.1.1 Extracellular plaques-consisting of beta amyloid

Amyloid plaques are extracellular deposits of Aβ that include abundant insoluble amyloid fibrils (7-10 nm) intermixed with nonfibrillar forms of the peptide (Selkoe, 1999). Cerebral amyloidosis occurs primarily in the neocortex and the hippocampus. Senile plaques also have been described in other brain regions like striatum, thalamus and cerebellum of AD patients, or even other neurodegenerative diseases (e.g. Parkinson disease and Huntington's disease) (Ozturk et al., 2002). Degenerative structure of neurons and abundant astrocytes and microglia can be associated with senile plaque deposits.

Aβ peptides, natural products of metabolism composed by 36 to 43 amino acids, are generated after sequential proteolytic cleavage of APP by two different proteases, β- and γ-secretase (Querfurth and LaFerla, 2010). APP is cleaved by beta-site APP cleaving enzyme-1 (BACE1) and β-secretase to produce the secreted sAPPβ ectodomain and the membrane-bound C-terminal fragment C99. Then, C99 is cleaved by γ-secretase, which releases $A\beta_{40}$ and $A\beta_{42}$ (Selkoe, 1998; Velliquette et al., 2005). The damaging and aggregation-prone $A\beta_{42}$ species are much less than monomers of $A\beta_{40}$ (Querfurth and LaFerla, 2010). An unbalance between clearance and production, and aggregation of peptides, lead to Aβ accumulation and amyloid plaques formation. After that, a series of biological events initiate which end up with an impairment of neuronal dendrites and synapses (Roberson and Mucke, 2006). This theory is called amyloid hypothesis (Querfurth and LaFerla, 2010), which is based on plentiful studies of AD genetic types (Busciglio et al., 2002), and evidences that $A\beta_{42}$ is toxic to cells (Selkoe, 2001; Tanzi and Bertram, 2005).

1.1.2 Neuro-inflammation

Besides well-known amyloid hypothesis, many recent researches came up the theory that neuro-inflammation also plays an integral role in the pathophysiology and progress

of the multifactorial disorder, facilitating Aβ deposition, neuronal loss, and cognitive deficits (Herrmann et al., 2011). Neuro-inflammation is characterized by release of numerous inflammatory mediators, microglial activation and astrogliosis, in particular around Aβ plaques (Akiyama et al., 2000; Wyss-Coray and Mucke, 2002). The inflammatory reaction in AD is a self-defense response in order to eliminate threatening stimuli and restore tissue structure integrity. However, when inflammation becomes excessive or chronic, it may lead to harmful consequences including excessively secretion of inflammatory mediators, further aggregation of amyloid peptides and neuronal dystrophy (Nussbaum and Ellis, 2003). Meanwhile, Aβ can stimulate the production of pro-inflammatory cytokines in glial cells such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), by setting up a vicious cycle (Rubio-Perez and Morillas-Ruiz, 2012). In a family study involving 206 middle-aged descendant of 92 families with a parental history of SAD and 200 descendant of 97 families without a parental history of SAD in the Netherlands (van Exel et al., 2009), increased levels of inflammatory markers (e.g. TNF-α, IL-1β and IL-6) was proven to be risk factors for developing AD in old age. The understanding of neuro-inflammation brings about a conception that anti-inflammatory drugs may be beneficial to neurodegeneration (Martin-Moreno et al., 2012b).

1.2 Epidemiology and genetics

AD prominently affects approximately 15% of the individuals older than 65 years and half of the population aged over 85 years (Smith and Whitehouse, 1998). Western Europe has the leading position in the prevalence of dementia in individuals older than 60 years (5.4%) after North American (6.4%), followed by Latin American (4.9%) and western-Pacific nations (4.0%). The annual incidence rates of regional dementia are estimated to be 10.5 per 1000 individuals for North American, 9.2 for Latin American, 8.8 for Western Europe, and 8.0 for East Asia (Ferri et al., 2005). However, as not all AD cases could be diagnosed by general practitioners and patients' relatives, the

incident cases might be even higher (Callahan et al., 1995; Ross et al., 1997). Moreover, as population life expectancy increases, in the Europe and USA, the number of affected persons is anticipated to triple by the middle of this century to 16.2 and 13.2 million, respectively (Hebert et al., 2003; Wancata et al., 2003).

AD can be subdivided into two different forms based on genetic predisposition and age of onset. Over 95% cases of AD come out after 65 years which are called late onset or sporadic AD (LOAD or SAD), while no more than 5% of AD onsets are manifested by age 60 and account for early-onset autosomal dominant forms, called familial AD (FAD) (Beach, 2008). Currently, AD research is primarily dependent upon the later form of disease. Two reasons can be proposed for explaining this. Firstly, FAD entirely embodies amyloid assumption that is the widely known and accepted theory describing molecular mechanisms of AD (Hardy and Allsop, 1991). Secondly and most importantly, transgenic models were designed considering mutated genes of familial forms, which are now of paramount importance to research.

Three genes are responsible to a large number of FAD cases, including the information encoding amyloid precursor protein (APP), presenilin1 (PS1) and presenilin2 (PS2). Their variations contribute to Aβ accumulation, which is more likely to form plaques (Bertram et al., 2010; Borchelt et al., 1996). No more than 3% of APP mutations are related to FAD, the majority of FAD mutations are found in the genes encoding PS1 and PS2. Mutations in PS1 are supposed to account for 30%-70% of FAD cases, while mutations in PS2 might take up no more than 5% (Bird, 1993). Children of a parent with FAD have a 50% chance of inheriting the mutation and developing the disease. Members of the family who do not inherit the mutations are no more like to get the disease than the other members of the general population (Nochlin et al., 1993).

1.3 APP/PS1 transgenic mouse model

As described above, the amyloid hypothesis primarily emphasizes that an excess of Aβ

production was the core pathology of AD (Hardy and Allsop, 1991). The presence of defined genes responsible for FAD has assisted making AD models overexpressing APP and PS1. These APP/PS1 transgenic mice represent major pathological features of AD, including parenchymal and vascular amyloid pathology, plaque-associated dystrophic neuritis, microglial activation, synaptic impairments, and learning and memory deficits (Li et al., 2013a).

Several APP/PS1 transgenic mice strains have been developed: APP/PS1 mice which express both human mutant APP (K670N/M671L) and human mutant PS1 (M146L) (Holcomb et al., 1998), mice expressing human mutant APP751 (KM670/671NL and V717I) and human mutant PS1 (M146L) (Schmitz et al., 2004), mice expressing human mutant APP (K670N/M671L + V717I) and human mutant PS1 (M233T/L235P) (Casas et al., 2004), mice harboring mutant APP (K594M/N595L) and PS1(A246Eor dE9) (Jankowsky et al., 2004), and mice harboring mutant APP (K670N/M671L + I716V + V717I) and PS1 (M146L+ L286V) also known as 5XFAD mice (Oakley et al., 2006). The greatest advantage of these models is exhibiting a rapid neuritic-type amyloid deposition at very early age, while at the same time Aβ deposition can be detected in the cingulate and motor cortex and hippocampus. Aβ accumulation accelerates with ageing (Holcomb et al., 1998), and the Aβ42/Aβ40 ratio increases accordingly (Borchelt et al., 1996; Oakley et al., 2006). These models are associated with cognitive deficits at early age and extensive neural loss, but not with NFTs (Oakley et al., 2006). As it is one of the most used mouse models, this model has been repeatedly used for the preclinical investigations (e.g. Acetyl-l-carnitine, 6D11 and All-trans retinoic acid) (Li et al., 2013a).

Recently, an APP/PS1-21 mouse model which co-expressed human mutant APP (KM670/671NL) and PS1 (L166P) was described (Tippmann et al., 2009) and employed in our current research. This transgenic starin combines many advantages of previous transgenic mice: First, mice were generated on a pure C57BL/6J background, which breed well and reduced the variability of Aβ metabolism and deposition;

Moreover, there was no obvious gender effect in the level of Aβ and amyloid deposition. In addition, APP/PS1-21 mouse could express a rapid neuritic-type amyloid deposition at very early age, thereby facilitating testing of therapeutic amyloid-targeting strategies.

1.4 Polyphenols

Natural polyphenols are most commonly found compounds in foods and consumed herbal beverages all over the world (Ramos, 2007). A plenty of evidence revealed that nature products, especially vegetables and fruits, could reduce the incidence of age-associated neurological diseases due to their high polyphenol content (Bastianetto et al., 2009). Epidemiological researches showed that the risk of developing dementia was lower in elderly people who regularly drank up to three glass of red wine per day (relative risk ranging from 0.55 to 0.58), consumed vegetables and fruits at least three servings per day (relative risk of 0.72), or drank vegetable and fruit juices more than three times per week (relative risk of 0.84). These findings from population surveys were supported by *in vivo* models of neurological disorders and *in vitro* models of toxicity, revealing that polyphenol-rich plant extracts expressed neuroprotective abilities or even reversed cognitive deficits (Bastianetto et al., 2000; Choi et al., 2001; Han et al., 2004; Joseph et al., 2003; Kwak et al., 2005; Stackman et al., 2003). Resveratrol, a polyphenol abundant in the skin of grapes, red wine, mulberries, and several types of nuts, inhibited $A\beta_{42}$ fibril formation and protected from Aβ neurotoxicity (Feng et al., 2009; Huang et al., 2011); moreover, it inhibited lipopolysaccharide (LPS)- stimulated production of inflammatory molecules (e.g. C-reactive protein) from primary mouse astrocytes (Wight et al., 2012). A flavone derivative 7,8-dihydroxyflavone (7,8-DHF) ameliorated AD-associated memory deficits in 5XFAD mouse brains, which owned to decreases in β-amyloidogenesis and BACE1 expression through activation of tyrosine receptor kinase B (Devi and Ohno, 2012). Rutin is a plant pigment (flavonoid) that is found in certain fruits and vegetables. Some research suggested that it not merely inhibited Aβ formation but also disaggregated Aβ fibrils (Jimenez-Aliaga et al., 2011), perhaps due

to attenuation of inflammatory cascade by decreasing cytokines, such as IL-1β and TNF-α (Wang et al., 2012). The ability of polyphenols to reduce Aβ toxicity by anti-inflammation and other mechanisms suggests their therapeutic potential against neurodegenerative diseases like AD (Bhullar and Rupasinghe, 2013).

1.4.1 Literature search

Through searching PubMed, we selected four polyphenols, namely Hesperidin, Icariin, Dihydromyricetin (DHM) and Baicalin, that possessed potential neuroprotective properties and listed their affecting protein targets in Table 1. Then, overlapping between known therapeutic targets and herbal targets were identified with the information from Therapeutic Target Database (TTD, http://bidd.nus.edu.sg/group/cjttd/, Department of Pharmacy, Faculty of Science, National University of Singapore, Singapore), which currently includes 388 approved successful (targeted by at least one approved drug), 461 clinical trial (targeted by drugs in clinical trial) and 1,467 research targets (targeted by experimental drugs only) (Chen et al., 2002; Zhu et al., 2010; Zhu et al., 2012).

Among the 48 herbal targets listed in the Table 1, 2 are approved successful anti-AD targets, 10 are successful targets for inflammation, neurodegenerative diseases and cancer, 31 are clinical trial or research targets for AD, inflammation, cardiovascular diseases and so on. These two approved successful anti-AD targets, named AChE and APP, were both inhibited by Icariin. BACE-1, c-Jun N-terminal kinase (JNK) and GSk-3β, which were characterized as clinical trial or research targets for AD, were also reported to be inhibited by Icariin and Baicalin. 6 of 10 another approved successful targets and 24 of 31 clinical trial or research targets were associated with neurodegenerative diseases and inflammation; and many of them were hit by more than one compound. Literature search showed their potential correlation with the process of AD. For instance, IL-6, PPARγ and COX-2 were supposed to be related with AD in a

great deal of studies (Cojocaru et al., 2011; Jiang et al., 2008a; Trepanier and Milgram, 2010). These results implied that these four polyphenolic compounds might be beneficial to AD. Detailed information about these compounds was described as follows.

Table 1 Target proteins of Hesperidin, Icariin, DHM and Baicalin

Target name (abbreviation)	Uniprot_ID	Compound	Effects	State of target*	Reference
Acetylcholinesterase (AChE)	P22303	Icariin	↓	Approved successful target (AD)	(He et al., 2010; Zhang et al., 2012b)
Amyloid beta protein (APP)	P05067	Icariin	↓	Approved successful target (AD)	(Jin et al., 2014; Zhang et al., 2014)
Cyclooxygenase-2 (COX-2)	P35354	Hesperidin	↓	Approved successful target (Cancer, inflammation and neurodegenrative disease)	(Hirata et al., 2005; Kamaraj et al., 2010; Sakata et al., 2003)
		Baicalin			(Cheng et al., 2012; Tu et al., 2009; Wang et al., 2006a)
		Icariin			(Chen et al., 2010b; Zeng et al., 2010a)
Interleukin-1β (IL-1β)	P01584	Hesperidin	↓	Approved successful target (Inflammation)	(Choi et al., 2007; Lee et al., 2011; Li et al., 2010a; Mahmoud et al., 2012; Raza et al., 2011; Visnagri et al., 2014)
		Icariin			(Zeng et al., 2010a)
		Baicalin			(Guo et al., 2013; Wang and Liu, 2014; Zhou et al., 2014)
		DHM			(Qi et al., 2012)
Nitric oxide synthase, Inducible (iNOS)	P35228	Hesperidin	↓	Approved successful target (Inflammation and ischemia reperfusion injuries)	(Ahmad et al., 2012; Raza et al., 2011; Sakata et al., 2003; Xiaoting et al., 2010)
		Icariin			(Chen et al., 2010b; Zeng et al., 2010a)
		Baicalin			(Feng et al., 2013; Tu et al., 2009);
		DHM			(Qi et al., 2012)
Peroxisome proliferator-activated receptor alpha (PPARα)	Q07869	Icariin	↑	Approved successful target (Cardiovascular disease)	(Ding et al., 2007)
Peroxisome proliferator-	P37231	Hesperidin	↑	Approved successful target	(Ghorbani et al., 2012)

activated receptor gamma (PPARγ)		Baicalin		(Cancer, inflammation, cardiovascular disease and neurodegnerative disease)	(Lim et al., 2012; Qiao et al., 2011)
Phosphodiesterase-5 (PDE5)	O76074	Icarin	↓	Approved successful target (Erectile dysfunction)	(Jin et al., 2014; Ning et al., 2006)
Serine/threonine-protein kinase mTOR (mTOR)	P42345	Baicalin	↓	Approved successful target (Cancer)	(Xia et al., 2014)
Signal transducer and activator of transcription 3 (STAT3)	P40763	Baicalin	↓	Approved successful target (Cancer)	(Xiong et al., 2013)
Tumor necrosis factor-α (TNF-α)	P01375	Hesperidin	↓	Approved successful target (Cancer and inflammation)	(Choi et al., 2007; Lee et al., 2011; Li et al., 2010a; Mahmoud et al., 2012; Raza et al., 2011; Visnagri et al., 2014; Yeh et al., 2007)
		Icariin			(Chen et al., 2010b; Wu et al., 2012; Wu et al., 2013b)
		Baicalin			(Guo et al., 2013; Li et al., 2012a; Liu et al., 2007; Yang et al., 2013)
		DHM			(Qi et al., 2012; Yang et al., 2012b)
Vascular endothelial growth factor A (VEGF-A)	P15692	Hesperidin	↓	Approved successful target (Cancer, inflammation and Ischemic heart disease)	(Shi et al., 2012a)
		Icariin	↑		(Xin et al., 2012)
		Baicalin	↓		(Chen et al., 2013a; Sun et al., 2013)
		DHM	↓		(Luo et al., 2006)
β-site APP-cleaving enzyme 1 (BACE-1)	P56817	Icariin	↓	Clinical trial target (AD)	(Jin et al., 2014; Zhang et al., 2014)
Apoptosis regulator BAX	Q07812	Hesperidin	↓	Research target	(Ahmad et al., 2012; Park et al., 2008; Tamilselvam et al., 2013)

(BAX)		Baicalin		(Cancer)	(Guo et al., 2014)
Apoptosis regulator Bcl-2 (Bcl-2)	P10415	Hesperidin	↑	Clinical trial target (cancer and neurodegenerative disease)	(Tamilselvam et al., 2013)
		Baicalin			(Guo et al., 2014)
Brain-derived neurotrophic factor (BDNF)	P23560	Baicalin	↓	Research target (neurodegnerative disease)	(Cao et al., 2011; Lee et al., 2014)
c-Jun N-terminal kinase kinase (JNK)	P45985	Icariin	↓	Clinical trial target (AD, diabetes mellitus and inflammation)	(Li et al., 2011; Zeng et al., 2014)
			↑		(Li et al., 2010b; Song et al., 2013)
		Baicalin	↓		(Hou et al., 2012; Luo et al., 2012)
Caspase-3 (CASP-3)	P42574	Hesperidin	↓	Research target (neurodegnerative disease)	(Ahmad et al., 2012; Hwang and Yen, 2008; Park et al., 2008; Wang et al., 2013a)
		DHM			(Ye et al., 2008)
		Baicalin			(Cao et al., 2011; Guo et al., 2014; Leung et al., 2007; Shu et al., 2014; Tu et al., 2009; Zheng et al., 2014)
		DHM			(Ye et al., 2008)
Caspase-9 (CASP-9)	P55211	Hesperidin	↓	Research target (neurodegenerative disease)	(Ahmad et al., 2012; Tamilselvam et al., 2013; Wang et al., 2013a)
		Icariin	↑		(Wang et al., 2011)
		Baicalin	↑		(Huang et al., 2012; Ma et al., 2005; Shu et al., 2014)
Extracellular signal-regulated kinase 1 (ERK1)	P27361	Hesperidin	↑	Research target (neurodegnerative disease)	(Chen et al., 2010a)
		Icariin	↓		(Hsieh et al., 2011; Li et al., 2013b)
			↑		(Nan et al., 2012; Song et al., 2013)
		Baicalin	↓		(Hou et al., 2012)
Extracellular signal-	P28482	Hesperidin	↑	Research target	(Chen et al., 2010a)

regulated kinase 2 (ERK2)		Icariin	↓	(neurodegenerative disease)	(Hsieh et al., 2011; Li et al., 2013b)
			↑		(Nan et al., 2012; Song et al., 2013)
		Baicalin	↓		(Hou et al., 2012)
Glycogen synthase kinase-3 beta (GSk-3β)	P49841	Icariin	↓	Clinical trial target (AD)	(Zeng et al., 2010b)
Glutathione synthetase (GSH)	P48637	Hesperidin	↑	Research target (neurodegenerative disease)	(Anandan and Subramanian, 2012; Tamilselvam et al., 2013)
Interleukin-2 (IL-2)	P60568	Hesperidin	↑	Research target (inflammation)	(Li et al., 2008)
Interleukin-5 (IL-5)	P05113	Hesperidin	↓	Research target (inflammation)	(Kim et al., 2011a)
Interleukin-6 (IL-6)	P05231	Hesperidin	↓	Clinical trial target (inflammation)	(Lee et al., 2011; Li et al., 2012b; Mahmoud et al., 2012; Yeh et al., 2007)
		Icariin			(Zeng et al., 2010a)
		Baicalin			(Guo et al., 2013; Lee et al., 2011; Liu et al., 2007; Luo et al., 2012; Ohtake et al., 2002; Wang and Liu, 2014; Yang et al., 2013; Zhou et al., 2014)
		DHM			(Qi et al., 2012)
Interleukin-8 (IL-8)	P10145	Hesperidin	↓	Research target (inflammation)	(Choi et al., 2007; Yeh et al., 2007)

		Baicalin	↓	Research target (inflammation)	(Luo et al., 2012)
Interleukin-10 (IL-10)	P22301	Hesperidin	↑	Research target (inflammation)	(Li et al., 2010a)
Interleukin-13 (IL-13)	P35225	Baicalin	↓	Research target (inflammation)	(Sun et al., 2013)
Interleukin-17 (IL-17)	Q9UHF5	Hesperidin	↓	Research target (inflammation)	(Kim et al., 2011a)
Mitogen-activated protein kinase, p38 (P38)	P53778	Icariin	↓	Clinical trial target (Cancer, inflammation, cardiovascular disease and neurodegenrative disease)	(Chen et al., 2010b; Li et al., 2011; Liu et al., 2011; Zeng et al., 2010a; Zeng et al., 2014)
			↑		(Ding et al., 2008; Ding et al., 2007; Hsieh et al., 2011; Mao et al., 2012b; Wang et al., 2009)
		Hesperidin	↓		(Kim et al., 2011b; Moon and Kim, 2012)
		Baicalin	↓		(Feng et al., 2013; Guo et al., 2013; Hou et al., 2012; Kim et al., 2006a; Luo et al., 2012; Wang et al., 2013b)
Nitric oxide synthase, endothelial (NOS3)	P29474	Icariin	↑	Clinical trial target (Cancer and inflammation)	(Chung et al., 2008)
Nuclear factor erythroid 2-related factor 2 (NRF2)	Q16236	Hesperidin	↑	Research target (Cancer)	(Chen et al., 2010a; Elavarasan et al., 2012)
Nuclear factor of kappa	Q04206	Hesperidin	↓	Research target	(Ahmad et al., 2012; Ghorbani et al., 2012; Kim et al., 2006b;

light polypeptide gene enhancer in B-cells 3 (NF-κB)				(inflammation)	Nazari et al., 2011; Yeh et al., 2009)
		Icariin			(Chen et al., 2010b; Hsieh et al., 2011; Li et al., 2014; Shi et al., 2014; Xu et al., 2011; Xu et al., 2010; Zeng et al., 2010a; Zhang et al., 2013a; Zhang et al., 2013b)
		Baicalin			(Chen et al., 2013a; Cheng et al., 2012; Guo et al., 2013; Kim et al., 2006a; Kim et al., 2008b; Lim et al., 2012; Lin et al., 2014; Lixuan et al., 2010; Luo et al., 2012; Xue et al., 2010; Yun et al., 2013; Zhou et al., 2014)
		DHM			(Qi et al., 2012; Yang et al., 2012b)
Phosphatidylinositol 3-kinase regulatory subunit alpha (PI3K)	P27986	Hesperidin	↑	Research target (Cancer)	(Nones et al., 2011)
		Icariin			(Chung et al., 2008; Xu et al., 2010; Zeng et al., 2010b; Zhang et al., 2012a)
		Baicalin	↓		(Nayak et al., 2014)
RAC-alpha serine/ threonine-protein kinase (PKB)	P31749	Icariin	↑	Clinical trial target (Cancer, neurodegenerative disease)	(Chung et al., 2008)
		Hesperidin			(Rong et al., 2013)
		Baicalin			(Kim et al., 2008b)

Toll-like receptor 2 (TLR2)	O60603	Baicalin	↓	Research target (Skin diseases)	(Guo et al., 2014; Li et al., 2012a; Lin et al., 2014; Tu et al., 2011b)
Toll-like receptor 4 (TLR4)	O00206	Baicalin	↓	Research target (cardiovascular diseases)	(Hou et al., 2012; Li et al., 2012a; Lin et al., 2014; Tu et al., 2011b; Yun et al., 2013)
Transcription factor AP-1 (AP-1)	P05412	Hesperidin	↓	Clinical trial target (Cancer and inflammation)	(Yeh et al., 2009)
		Icariin	↑		(Wo et al., 2008)
Transforming growth factor-β (TGF-β)	P01137	Hesperidin	↓	Research target (Cancer)	(Yang et al., 2012c)
		Icariin			(Li et al., 2013b; Qi et al., 2011)
		Baicalin			(Qiao et al., 2011; Sun et al., 2013)
Tumor suppressor p53 (p53)	P04637	Hesperidin	↑	Clinical trial target (Cancer)	(Ghorbani et al., 2012)
		Icariin	↑		(Zhu et al., 2005)
			↓		(Li et al., 2011)
		Baicalin	↓		(Guo et al., 2014; Leung et al., 2007)
		DHM	↑		(Wu et al., 2013a)
Tyrosine-protein kinase JAK2 (JAK)	O60674	Baicalin	↓	Clinical trial target (inflammation and cardiovascular disease)	(Xiong et al., 2013)

Heme oxygenase 1 (HMOX1)	P09601	Hesperidin	↑	none	(Chen et al., 2010a)
Matrix metalloproteinase-2 (MMP-2)	P08253	Hesperidin	↓	none	(Kamaraj et al., 2010)
		Icariin			(Song et al., 2011)
		Baicalin			(Wang et al., 2013b)
Matrix metalloproteinase-9 (MMP-9)	P14780	Hesperidin	↓	none	(Kamaraj et al., 2010; Yeh et al., 2009)
		Icariin	↓		(Song et al., 2011)
		Baicalin	↓		(Tu et al., 2011a; Wang et al., 2013b; Zhou et al., 2014)
Matrix metalloproteinase-13 (MMP-13)	P45452	Icariin	↓	none	(Zeng et al., 2014)
Nuclear respiratory factor 1 (NRF-1)	Q16656	Icariin	↑	none	(Ding et al., 2007)
Transcription factor Sp1 (Sp1)	P08047	Hesperidin	↓	none	(Lee et al., 2012)

*Successful target: targeted by at least one approved drug; Clinical trial target: targeted by drugs in clinical trial; Research targets: targeted by experimental drugs only; None: target was not found in the TTD database.

1.4.2 Description of Hesperidin, Icariin, DHM and Baicalin

(1) Hesperidin

Hesperidin ($C_{28}H_{34}O_{15}$, Figure 1) is a naturally occurring bioflavonoid and is one of the two molecules which were erroneously named 'Vitamin P' previously (Garg et al., 2001). It was first isolated in 1828 by French chemist Lebreton from the *albedo* (the spongy inner portion of the peel) of oranges, and has since been found in lemons and other citrus fruits (Manthey and Grohmann, 1998). Hesperidin concentration appears to be high in the *Citrus sinensis* (15.25±8.21 mg/100g fresh fruit weight) and *Citrus reticulate* (19.26±11.56 mg/100g fresh fruit weight) (Peterson et al., 2006). In a recent Finland survey on polyphenol intake, Hesperidin was revealed to account for approximately 30% of the total flavonoid intake, with a high consumption of 28.3 mg every day (Knekt et al., 2002). Also, it is well-known, that Hesperidin is one of the primary constituents of Chenpi, which is made of *Satsuma mandarin* peel and has traditionally been prescribed as a Traditional Chinese Medicine (TCM) for inflammation, allergy and hepatopathy (Yamada et al., 2006).

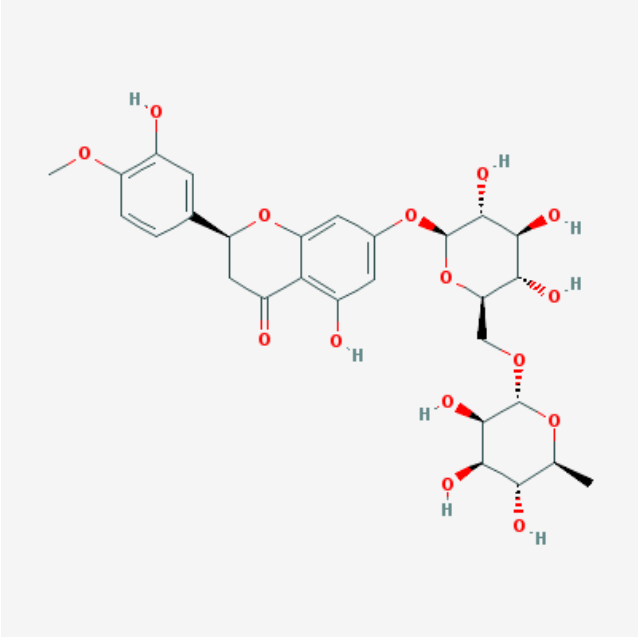


Figure 1 Molecular structure of Hesperidin

(http://pubchem.ncbi.nlm.nih.gov)

Hesperidin has pleiotropic biological activities and properties, and has been used in a wide range of diseases and disorders, including neurological disorders, psychiatric disorders and cardiovascular diseases (Garg et al., 2001). Daflon, a purified micronized flavonoid fraction containing Hesperidin and another flavonoid Diosmin, has been widely prescribed for treating venous circulation disorders (Geroulakos and Nicolaides, 1994) and acute hemorrhoidal attack (Cospite, 1994), owing to its multiple therapeutic activities (Ramelet, 2001; Roztocil et al., 2003). Interestingly, anti-inflammatory activity of Hesperidin and Daflon was also reported and considered to be crucial for their therapeutic values (Lyseng-Williamson and Perry, 2003). Hesperidin exerted anti-inflammatory activity in various animal models and cell types, indicated by a reduction of the production of pro-inflammatory cytokines (Li et al., 2010a; Yamamoto et al., 2013). In a double-blind crossover clinical trial, the increased circulating levels of pro-inflammatory markers (high-sensitivity CRP, serum Aβ protein and soluble E-selectin) in patients with metabolic syndrome were attenuated by hesperidin (Rizza et al., 2011). Considering the Hesperidin's capability of traversing the blood-brain barrier (BBB), it might also have inhibiting activity of neuro-inflammation. Indeed, it significantly reduced microglia and astrocyte activation, and attenuated neurobehavioral dysfunction in some neurodegenerative diseases, including Parkinson's disease (PD) and Huntington's disease (HD) (Menze et al., 2012).

(2) Icariin

The genus *Epimedium* with about 20 species is widely distributed in the temperate regions of Europe, North America and Asia (Kuroda et al., 2000). As the whole plant or folioles of some *Epimedium* species, *Epimedium* herb have been utilized for more than one thousand years to treat chronic nephritis, osteoporosis, asthma, cardiovascular problems, and hepatitis in East Asia (Chen et al., 2010b). Colloquially known as *yin yang huo* or *horny goat weed*, *Epimedium* is particularly interest for its perceived efficacy in the management of sexual concerns (Shindel et al., 2010).

Several components of the plants have been studied, but Icariin ($C_{33}H_{40}O_{15}$, Figure 2)

was identified as the most metabolically active extract of *Epimedium* that had anti-inflammatory properties, which down-regulated PGE(2), TNF-α and nitric oxide (NO), as well as inhibited the activation of NF-κB p65 (Zhou et al., 2011). Icariin was also proved to act as a neuroprotectant through inhibiting pro-inflammatory and pro-oxidant markers in response to stress (Li et al., 2011; Liu et al., 2011). Wang et al.'s research indicated the neuroprotective effect of Icarrin against $A\beta_{25-35}$ insult in primary cultured rat cortical neuronal cells (Wang et al., 2007b). *In vitro* Icariin protected neurons against cerebral ischemia/reperfusion via enhancing anti-oxidant capacity, decreasing cell apoptosis and preventing intracellular calcium concentration elevation (Li et al., 2005). Besides, Icariin increased anti-oxidant capacity, and decreased lipid peroxidation and $A\beta_{1-40}$ levels in the rat hippocampus, thereby improving the spatial learning and memory of aluminium-intoxicated rats (Luo et al., 2007).

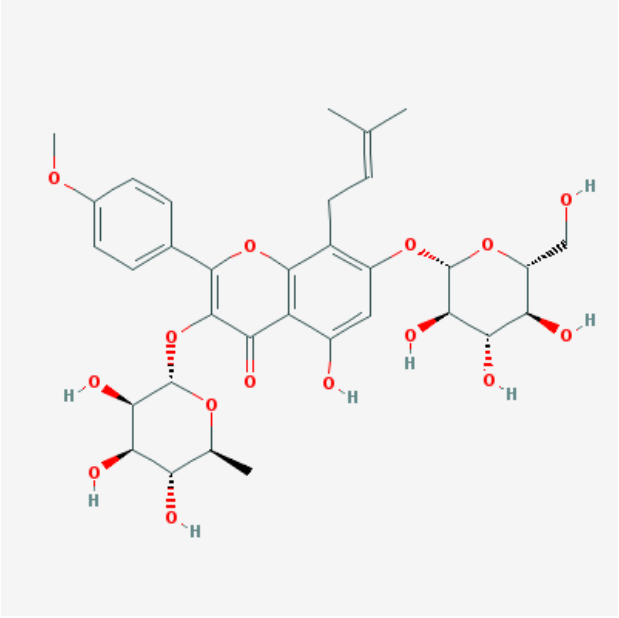


Figure 2 Molecular structure of Icariin

(http://pubchem.ncbi.nlm.nih.gov)

(3) Dihydromyricetin

Hovenia dulcis, as the premier anti-hangover herbal medicine, was listed in *Tang* Materia Medica, the China's first pharmacopeia published in the year of 659. Its extracts

ameliorated alcohol-induced liver injuries (Du et al., 2010), and relieved hangover, partly by promoting ethyl alcohol (EtOH) elimination thtough augment of acetaldehyde dehydrogenase (ALDH) and alcohol dehydrogenase (ADH) activity (Chen et al., 2006; Shen et al., 2012).

Dihydromyricetin (DHM, $C_{15}H_{12}O_8$, Figure 3), also known as Ampelopsin, is a flavanonol, a type of flavonoid. It is a natural supplement derived from *hovenia dulcis* tree. DHM has been reported to inhibit nitric oxide (NO) production in LPS-induced RAW264.7 cells and ameliorate carrageenan-stimulated acute inflammation *in vivo* (Ku et al., 2008). Previous study showed that DHM protected PC12 cells from H_2O_2-induced apoptosis (Kou et al., 2012) via activation of Serine/threonine kinase (Akt) and Extracellular signal-regulated kinase (ERK) signaling pathways. In sodium pentobarbital-induced mouse hypnosis experiments, it also increased activity of liver microsomal enzyme and accelerated metabolism of alcohol or sodium pentobarbital, thereby significantly extending the incubation period caused by sodium pentobarbital, reducing the length of time associated with hypnotic effects, and significantly reducing ethanol-induced inebriation reaction (Kou and Chen, 2012).

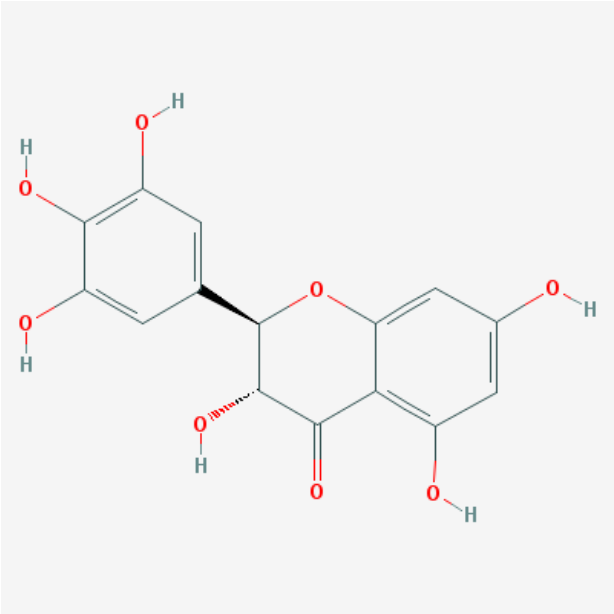


Figure 3 Molecular structure of Dihydromyricetin

(http://pubchem.ncbi.nlm.nih.gov)

(4) Baicalin

Huangqin, the roots of *Scutellaria baicalensis*, is one of the most popular traditional medical herbs in Asia and also officially listed in the Chinese Pharmacopoeia (Wu et al., 2005). In the past centuries, it is reputed to be effective in treating bleeding disorders (e.g. hematemesis, hematuria, and metrorrhagia) and cardiovascular disease (Chen et al., 2013b; Yoon et al., 2009).

Baicalin ($C_{21}H_{18}O_{11}$, Figure 4) is one of the main bioactive flavone glucuronides derived from *Huangqin*, and it is extensively used in the treatment of fever, inflammation, and other conditions (Zhao et al., 2013). During ischemia, Baicalin was known to preserve Heat Shock Protein 70 (HSP70) concentration, preserve ERK phosphorylation (cytoprotective) and reduce the phosphorylation of p38 MAPK and JNK (positively suppresses cell death), bringing about cytoprotective effects (Dai et al., 2013; Han and Holtzman, 2000; Xia et al., 1995). It also showed anxiolytic effects in a rat conflict test, which attributed to binding to the benzodiazepine binding site of the neurotransmitter gamma-aminobutyric acid (GABA)A receptor (Liao et al., 2003). Moreover, Baicalin and *Scutellaria baicalensis* have shown memory promoting and

anti-amnesiac effects against Aβ proteins (Kim et al., 2008a), chronic lipopolysaccharide infusion (Hwang et al., 2011), ibotenic acid (toxin that mimicks Alzheimer's) (Heo et al., 2009; Malek et al., 2009), γ-irradiation (isolated Baicalin) (Oh et al., 2013), ischemia (Shang et al., 2005), and in animal models of aging (Jeong et al., 2011; Shang et al., 2001; Song et al., 2009).

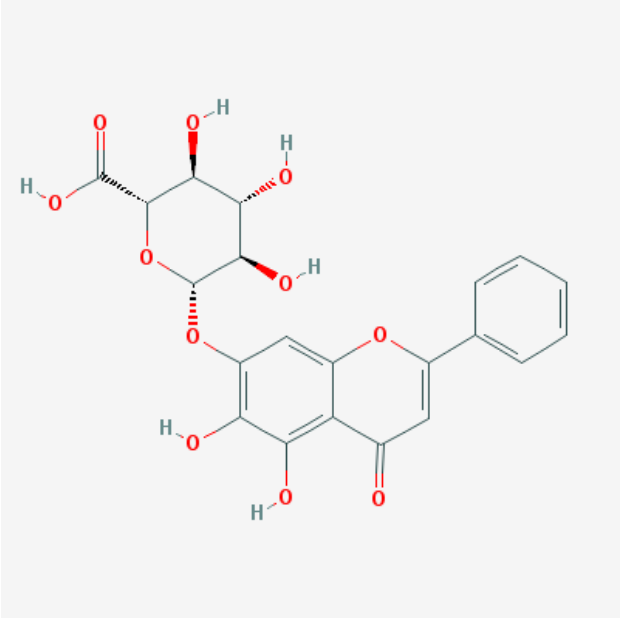


Figure 4 Molecular structure of Baicalin

(http://pubchem.ncbi.nlm.nih.gov)

1.4.3 Target network analysis of molecular mechanism for Hesperidin, Icariin, DHM and Baicalin

For purpose of elaborating the probable molecular mechanism of anti-AD for these four polyphenols, through cooperating with Life Science and Technology School, Tongji University, China, the possible polyphenol-target related proteins were assessed by the *in silico* ligand-protein inverse docking program INVDOCK (Chen and Zhi, 2001). For example, the conformation of Hesperidin molecule binding to BACE1 and TNF was shown in Figure 5.

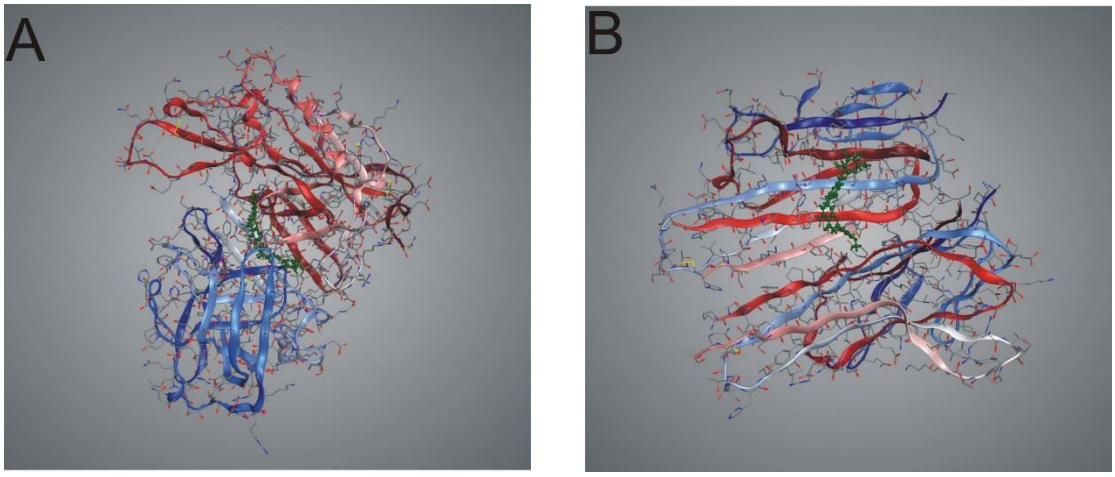


Figure 5 Illustration of Hesperidin molecule docked in BACE1 and TNF-α by INVDOCK program. (A) Docking model of the complex Hesperidin-BACE1; (B) Docking model of the complex Hesperidin-TNF-α. The Hesperidin molecule is displayed in ball and stick; the protein is displayed in ribbon model.

In all 16, 12, 17 and 18 proteins were respectively predicted as the potential AD-related targets for Hesperidin, Icariin, DHM and Baicalin through virtual docking, and involved in the "Alzheimer's disease pathway" from Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (Kanehisa and Goto, 2000; Ogata et al., 1999). As shown from Figure 6 to Figure 9, some AD-associated processes were concerned, such as amyloid pathway and inflammation.

According to the "amyloid hypothesis" mentioned above, Aβ peptides are produced through the endoproteolysis of APP. Cleavage of APP by β-secretase, also known as aspartic protease BACE1 in the first place and by γ-secretase in the second place, with PS1 or PS2 as the catalytic components, is required to liberate Aβ from APP (Selkoe, 1998; Velliquette et al., 2005). In contrast, α-secretase, which is the metalloprotease ADAM10, cuts APP within the Aβ domain, thus preventing Aβ formation (Lichtenthaler, 2012; Vassar et al., 2009). In addition, beta amyloid peptides are proteolytically degraded by insulin degrading enzyme (IDE) and neprilysin (NEP), which are remarkably enhanced by ApoE (Jiang et al., 2008b).

Intracellular accumulation of Aβ initiates a chronic inflammatory response in the cerebral cortex which is suspected to gradually exacerbate the disease (Rogers et al., 1996; Sastre et al., 2006). ERK 1/2 are localized in the cytoplasm, and are activated by phosphorylation. The activation of ERK 1/2 is followed by increased NF-κB activation and TNF-α secretion, thereby playing the dominate role in the inflammation and contributing to the AD process (Maeng et al., 2006). Previous research has reported that reducing ERK 1/2 phosphorylation and NF-κB activation suppressed inflammation such as TNF-α secretion (Indra et al., 2013).

From Figure 6 to Figure 9, all these targets described above were potential targets of these four polyphenols and hit AD pathway; they were highlighted with red boxes. It was suggested that Hesperidin, Icariin, DHM and Baicalin might produce anti-AD effects mainly through anti-amyloidosis and anti-inflammation. However, this hypothesis was needed to be certified through further studies.

The current research was designed to evaluate the potential therapeutic effect of these four polyphenols (Hesperidin, Icariin, DHM and Baicalin) on Aβ deposition, neuro-inflammation and behavioral dysfunction in the transgenic APP/PS1-21 mouse model, which is a widely applied animal model of cerebral amyloidosis and neuro-inflammation.

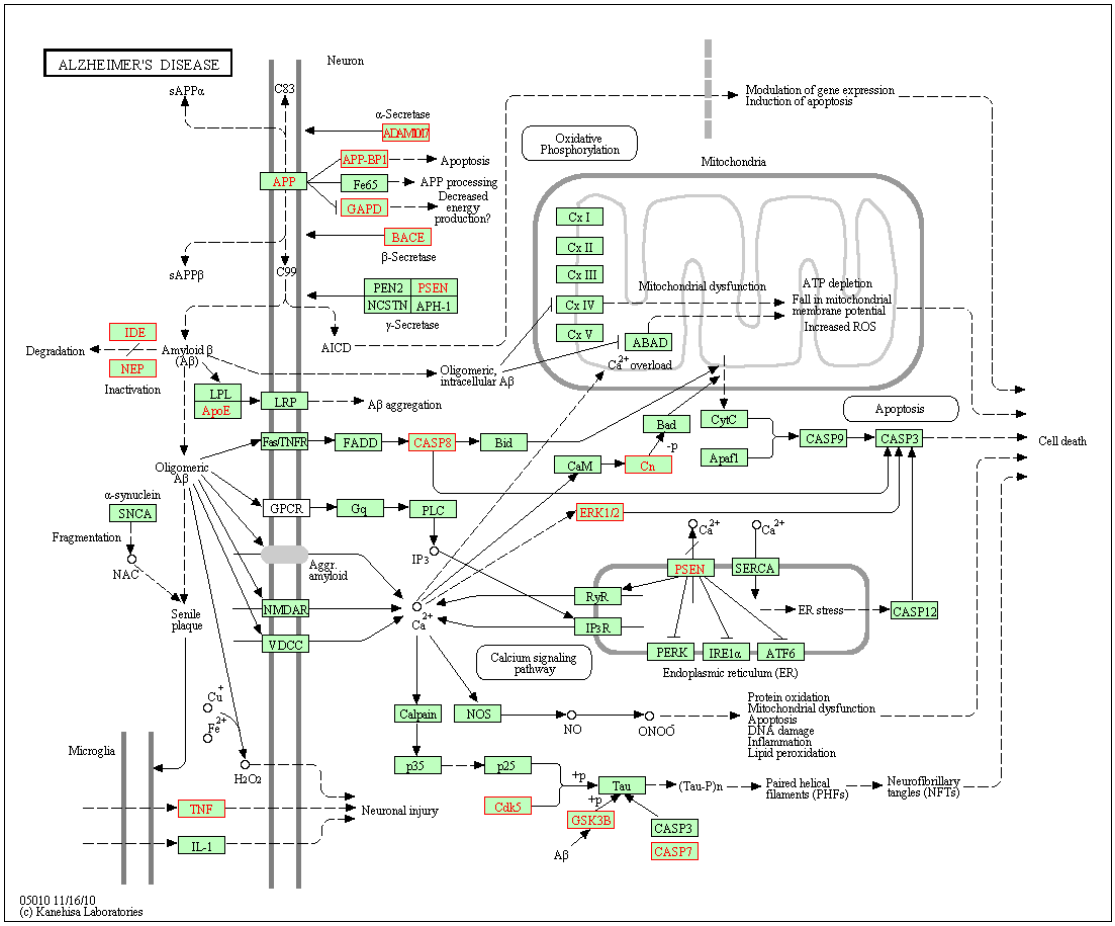


Figure 6. Distribution of target proteins of Hesperidin on "Alzheimer's disease pathway". Potential targets of Hesperidin hit AD pathway were highlighted with red boxes.

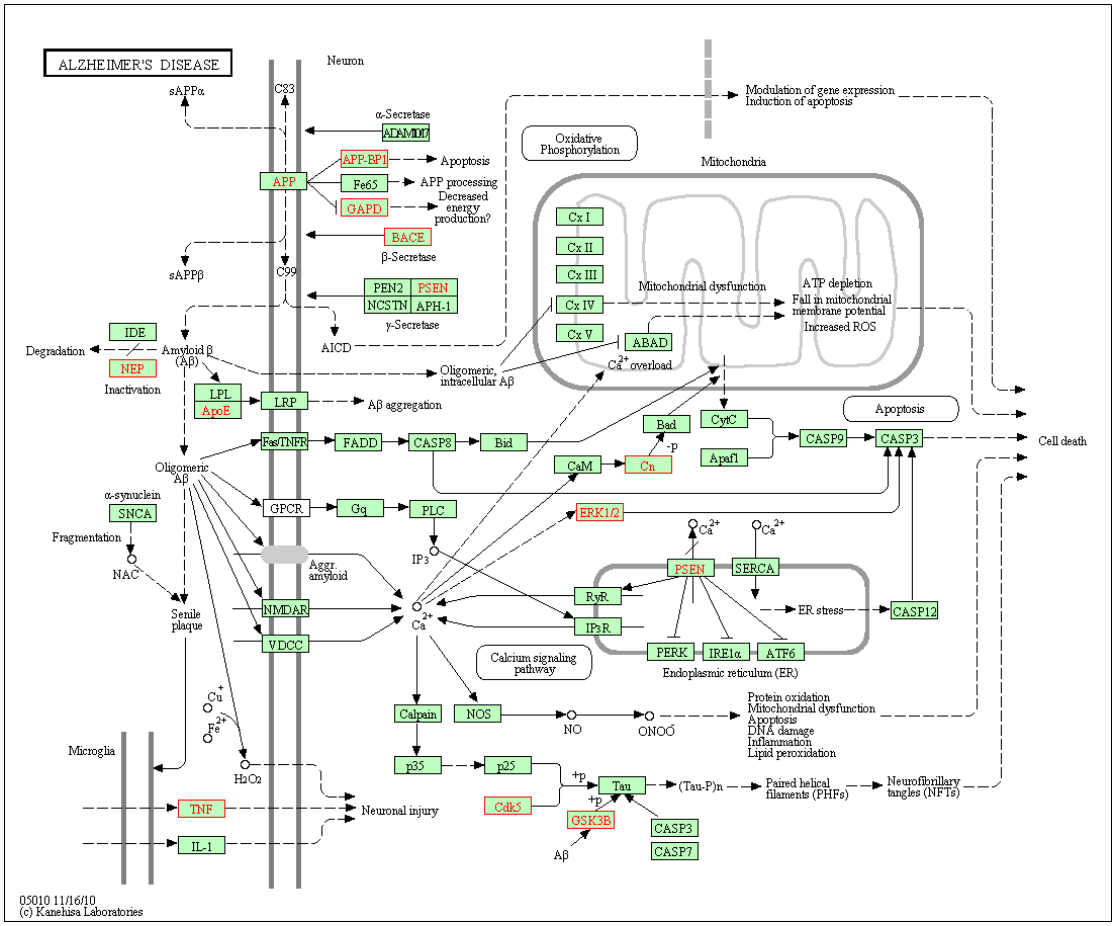


Figure 7. Distribution of target proteins of Icariin on "Alzheimer's disease pathway". Potential targets of Icariin hit AD pathway were highlighted with red boxes.

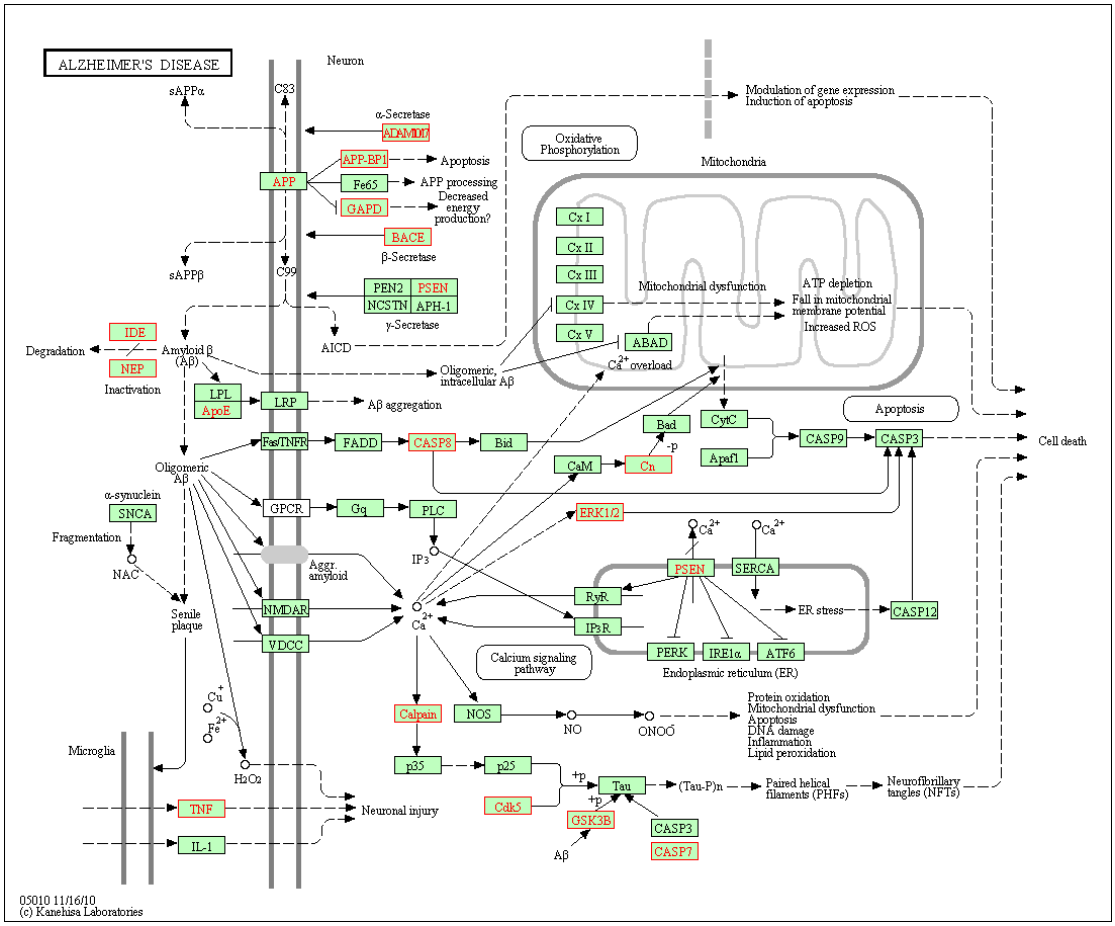


Figure 8. Distribution of target proteins of DHM on "Alzheimer's disease pathway". Potential targets of DHM hit AD pathway were highlighted with red boxes.

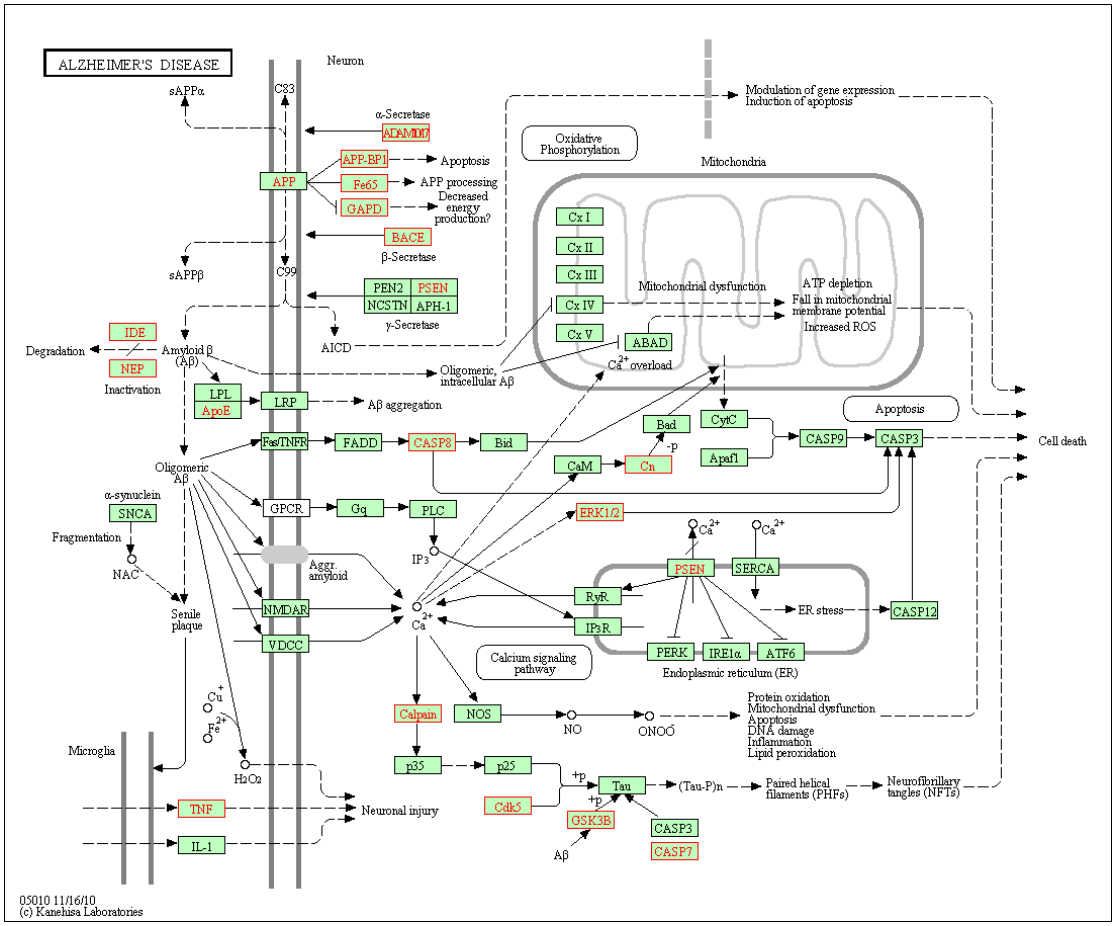


Figure 9. Distribution of target proteins of Baicalin on "Alzheimer's disease pathway". Potential targets of Baicalin hit AD pathway were highlighted with red boxes.

Chapter II: Methods and materials

2.1 Transgenic mice

In the current research we used transgenic APP/PS1-21 mice as a model of AD. These mice, which overexpress human APP (K670N/M671L) and PS1 (L166P) mutations on the congenic C57BL/6J background (Charles River Germany, Sulzfeld Germany), were obtained from Prof. M. Jucker and were bred with female C57BL/6J mice (Charles River Germany, Sulzfeld Germany) to yield offsprings. All offsprings were characterized by PCR genotyping with primers specific for the APP-sequence (Forward:"GAATTCCGACATGACTCAGG", Reverse: "GTTCTGCTGCATCTTGGACA"). Mice were kept in a quiet environment under a 12:12-h light/dark cycle. Accessible water and food was provided ad libitum. All experiments were licensed according to the The German Animal Welfare Act (TierSchG) of 2006.

2.2 Materials

Hesperidin, Icariin, DHM and Baicalin (all >98%) were purchased from Huike Botanical Development Co., Ltd (Xi'an, P.R. China), MR Natural Product Co., Ltd. (Xi'an, P.R. China), Lyphar Biotech Co., Ltd (Xi'an, P.R. China) and Tokyo Chemistry Co., Ltd (Tokyo, Japan), respectively. All of them were suspended in 1% carboxymethylcellulose (CMC, Blanose®, Hercules-Aqualon, Düsseldorf, Germany) and administered orally in a dose of 100 mg/kg (12.5 mg/ml). Control group animals received the same volume of solvent.

2.3 Treatment

The purpose of this research was to evaluate the neuroprotective effects of four polyphenols (Hesperidin, Icariin, DHM and Baicalin) on AD transgenic mice. However, as it was not possible to obtain so many mice at a time, the experiments were conducted sequentially for three times

In experiment I, two groups of animals were used.

Hesperidin group: six APP/PS1-21 mice, at age of 5 months, received Hesperidin treatment with a dose of 100mg/kg by daily gavage;

Control I: six gender-, age- and bodyweight matched APP/PS1-21 mice, as control, received the same volume of 1% CMC dissolved in water.

In experiment II, 18 mice were divided into three groups.

Icariin group: six APP/PS1-21 mice, at age of 5 months, received Icariin treatment with a dose of 100mg/kg by daily gavage;

DHM group: six gender-, age- and bodyweight matched APP/PS1-21 mice received DHM treatment with a dose of 100mg/kg by daily gavage;

Control II: another six transgenic littermates, as control, received the same volume of 1% CMC dissolved in water.

In experiment III, two groups of mice were used.

Baicalin group: six APP/PS1-21 mice, at age of 5 months, received Baicalin treatment with a dose of 100mg/kg by daily gavage;

Control III: six gender-, age- and bodyweight matched APP/PS1-21 mice, as control, received the same volume of 1% CMC dissolved in water.

2.4 Nest-building assay

Nest-building assay was performed as reported previously (Wesson and Wilson, 2011a). It was modified to determine the potential changes of affiliative social behavior of transgenic mice following treatment.

APP/PS1 mice were kept for 1 day in single plastic cages with about 1cm of wood chip bedding lining the floor and identification cards coded to make the investigators blind to grouping of mice. Two hours prior to the onset of the dark phase of the light cycle, squared small pieces of paper towel (5×5cm) were introduced inside the separate cages.

The presence and quality of the nest construction was evaluated in the next morning by a 3-point scale from 1 to 3. Score of 1: the paper was not noticeable torn or bitten; 2: the paper was moderately torn up, but not grouped into identifiable nest in a corner; 3: The great mass of paper was torn into pieces and gathered in a corner of the cage. Nest scores were given by three independent observers blinded to treatment categories and pictures were taken for documentation.

2.5 Social interaction assay

The social interaction assay was performed according to previous studies (Bolivar et al., 2007; Hibbits et al., 2009) with minor modifications. As a broad screen of activities, the resident-intruder assay was video-recorded to quantify all distinct behaviors of control and polyphenol-treated mice as a resident in the absence and presence of an intruder mouse, combined with analysis of movement to evaluate overall activity level and overt neurobiological differences. The chamber employed for testing the mouse interactive and independent behavior was a plastic cage (325mm x 210mm x 185mm), which was identical with our standard housing cages. Each transgenic mouse was introduced in this cage and permitted to run freely for 15 min. Then, a prepared gender-, age- and weight-matched non-treated naïve mouse was placed for a second 15 min session. Thus, the first transgenic mouse should be the 'resident' mouse of this cage, while the naïve mouse was the 'intruder' one. All behaviors during both two 15 min sessions were videotaped with a camera given a certain frame rate of 15 Hz. The frame rate made sure that rapid movements of the mice could be amply captured and in consideration of a close-grained analysis of the trajectory, yet was sufficient small for manageable document size.

Through playing back those videotapes, behavioral events of resident mice were counted by three independent observes blinded to group assignment. The interactive behavioral events included sniffing the other mouse, following, rearing at the other mouse, grooming, laying or sitting beside the other mouse, backing or running away

from the other mouse, boxing, biting, or wrestling, pinning, mounting, and tail-rattling. These independent behavioral events included sniffing the environment, rearing alongside the cage, digging, rearing independently, circling clockwise or counter-clockwise, freezing, allogrooming, and scratching (Hibbits et al., 2009).

For calculating the total distances traveled and scoring all identifiable distinct behaviors, both 15 min sessions were record by camera at a frame rate of 15 Hz. The region of interest in the captured video of size 500×310 pixels was saved directly to a computer for later analysis. Following acquisition of the video, the position of the mouse was tracked in each frame. Tracking was carried out in the computing environment Java using ICY software (de Chaumont et al., 2012). For determining the position of the mouse, the pixel of maximum intensity was detected in every frame, and a subset image circling this pixel was picked up. The center of intensity of the subset image was computed and used to record the mouse X and Y locations. Then, the total distance traveled per transgenic mouse could be easily calculated using the mouse behavior analysis software developed in our lab.

2.6 Immunohistochemistry (IHC) and image evaluation/analysis

Polyphenol-treated and control mice were sacrificed after the 10-days treatment. Mice were deeply anesthetized with CO_2 and sacrificed. Then, their brains were taken and post-fixed in PBS containing 4% paraformaldehyde overnight at 4 ℃. Post-fixed brains were cut into two hemispheres. After dehydration with alcohol, hemispheres were routinely processed in paraffin and serially sectioned at 3 μm. Then, brain sections were deparaffinized, rehydrated, and washed in PBS. After 15 min of incubation in 1% H_2O_2 to prevent endogenous peroxidation, the sections were blocked with 10% normal pig serum (Biochrom, Berlin, Germany) and then incubated with the following monoclonal antibodies: β-amyloid (1:100; Abcam, Cambridge, UK) for Aβ deposition, Iba-1 (1:200; Wako, Neuss, Germany) for activated microglia and GFAP (1:500; Chemicon (Millipore), Billerica, MA, US) for astrocytes. After these tissue sections were washed

3 times with PBS for 5 min, antibody binding to them was visualized with a biotinylated IgG $F(ab)_2$ secondary antibody (DAKO, Hamburg, Germany). Afterwards, these sections were detected with a Streptavidin–Avidin–Biotin complex (DAKO, Hamburg, Germany) and completed by using diaminobenzidine (DAB) substrate (Fluka, Neu-Ulm, Germany), followed by counter-staining with Maier's Hemalum (Zhang et al., 2008).

After immunostaining, tissue sections were examined by light microscopy (Nikon Coolscope, Nikon, Düsseldorf, Germany). Aβ deposition, Iba-1 and GFAP immunostaining were evaluated at cross-sections of hemispheres, especially focused on cortex and hippocampus. All sections were randomly numbered and analyzed by two observers independently, who were not aware of the treatment. Aβ plaques, Iba-1 and GFAP positive cells in cortex and hippocampus were manually counted, by a certain diameter and clear deposition for plaques, and cellular nuclear for cells.

To further evaluate immunostaining data, the percentages of areas of specific immune-reactivity (IR) in interesting regions were calculated. Briefly, images of hemisphere cross-sections were captured under 5×magnification using Nikon Cool-scope (Nikon, Düsseldorf, Germany) with fixed parameters; the cortex and hippocampus were manually outlined and further analyzed using the software MetaMorph Offline 7.1 (Molecular Devices, Toronto, Canada). Areas of IR were selected by color threshold segmentation and all parameters were fixed for all images. Results were given as arithmetic means of plaque/cell counts or area percentages of IR to interest areas on cross-sections and standard errors of means (SEM).

2.7 Statistical analysis

The data were calculated and expressed as means ± SEM. The differences of behavioral events, plaque/cell counts or IR area percentages between means of Hesperidin and control groups, or Baicalin and control groups were analyzed by unpaired student's

two-tailed t-test. All the parameters described above among Icariin, DHM and their control groups, were compared using one-way Analysis of Variance (ANOVA), followed by Dunnett's multiple comparison tests. Statistical significance was defined as P values<0.05. All statistical analyses were performed with the Graph Pad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA).

Chapter III: Results

3.1. Remediation of affiliative behavior impairment of APP/PS1 mice (nest construction assay)

As a born instinct, nesting behavior is of great importance for mice in heat conservation, reproduction and shelter. Our previous study showed that nesting ability of these transgenic mice was impaired compared to naive mice (Zhang and Schluesener, 2013). Prior to treatment, nest building performance in these four polyphenol-treated groups was not significant different as compared to vehicle-treated mice (Figure 10A, 10C and 10E). After the 10-days treatment (Day 11), nests built by Hesperidin- and Icariin-treated mice were of improved quality as indicated by significant differences in nesting scores as compared to their control counterparts, while the nesting scores in DHM- and Baicalin-treated groups were not obviously changed in comparison to their respective control groups (Experiment I: control I=1.4±0.2, Hesperidin=2.1±0.2, P=0.02, Figure 10B; Experiment II: control II=1.5±0.1, Icariin=2.1±0.4, DHM=1.8±0.2, P<0.01, Figure 10D; Experiment III: control III=1.4±0.2, Baicalin=1.3±0.2, P=0.76, Figure 10F). Moreover, relatively immediate chewing and tearing behaviors on the paper towels were observed in Hespeiridn and Icariin-treated mice; paper towels were torn into pieces and grouped into a corner. In sharp contrast, the DHM- and Baicalin-treated, and control animals investigated and slightly chew but did not really destruct the paper towels, just similarly as they did 10 days ago; paper towels were found all over in the cage, not grouped, or were grouped but not in the corner.

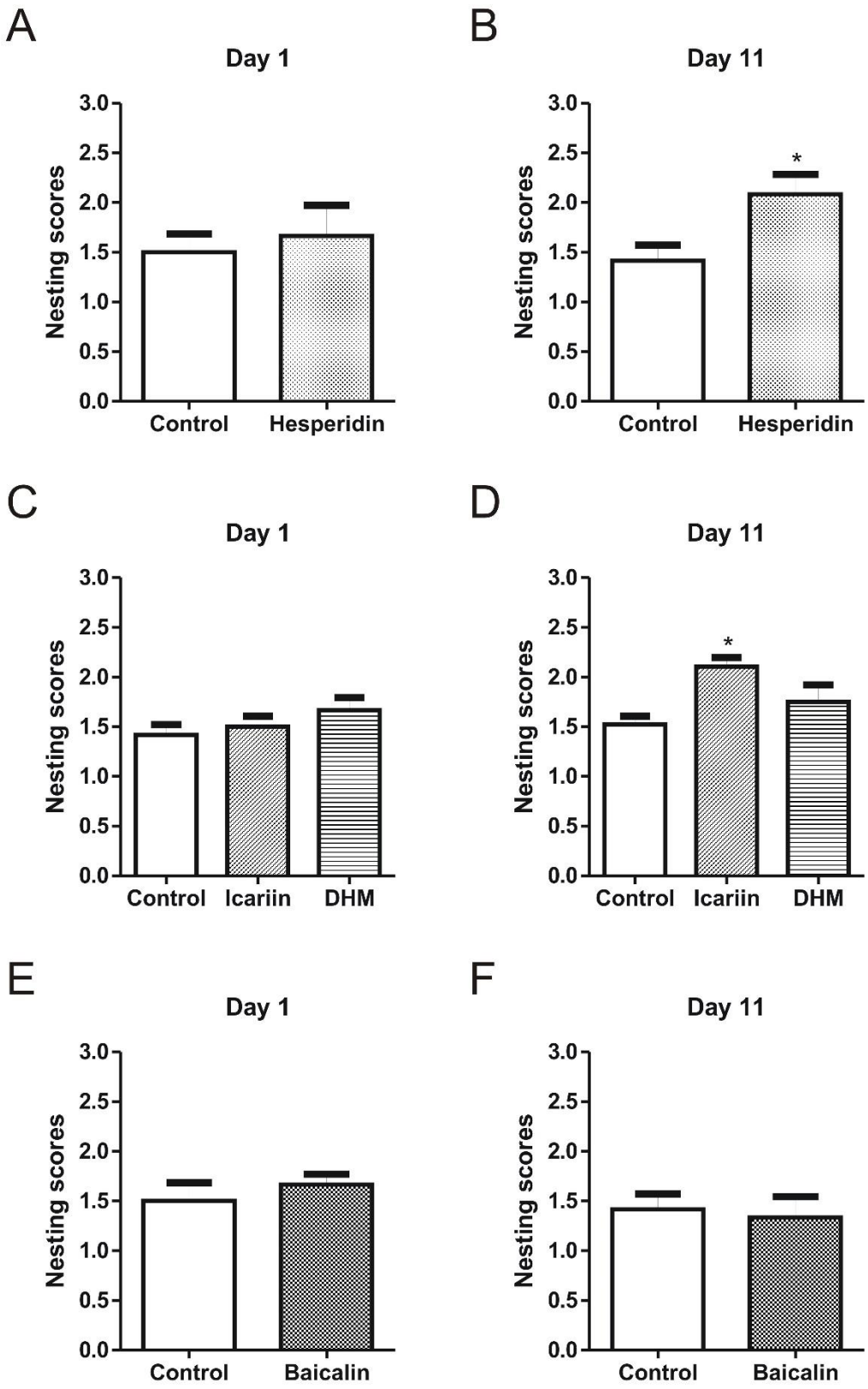


Figure 10. Effect of four polyphenols on impaired nesting ability. APP/PS1 mice received 10-days treatment of polyphenols or control by gavage. They were assessed for nesting

behavior and nest construction was explored with paper towel material using a 3 point scale. (A) No significant difference between Hesperidin and control group could be observed right at the beginning of treatment, namely at Day 1. (B) At Day 11, nest building score was significantly higher in mice treated by Hesperidin, compared to control mice. (C) Prior to treatment, there were no obvious difference among Icariin-, DHM-treated and control mice. (D) After 10 days treatment, the difference in the nesting scores among three groups were statistically significant; multiple comparison showed that the nesting score of Icariin group was notably higher than control group, while there was no significant difference between DHM and control group. (E and F) No matter prior to or after treatment, the difference in the nesting scores between Baicalin and control group was not significant. $^{*}P<0.05$ compared with control group.

3.2. Remediation of social interaction impairment of APP/PS1 mice (resident-intruder assay)

In the social interaction assay, all movements of transgenic mice were recorded by two cameras, one vertically placed camera for calculating the distances of movement and another from the side of cages for counting the individual and interactive behaviors. Both cameras were adjusted to proper height, which ensures that all animal movements could be captured.

Our data consisted of coordinates of the moving animal (considered as a point) sampled at 15 Hz. These calculated distances traveled using recorded X, Y coordinates were transformed to the real distances of movement (pixel to cm). No matter before or after treatment, the distances traveled were not statistically significantly different compared polyphenol-treated mice with their respective control mice (Figure 11).

Two unfamiliar mice placed in a same cage will often display high levels of sniffing, following, grooming, rearing at the other mouse, sitting or laying next to the other mouse and so on. The researchers scored the video sessions for frequency of carefully defined behavioral events. Prior to treatment, the difference of interactive behavior counts was not statistically significant compared polyphenol-treated mice with their

respective control mice, while following 10 days treatment, resident APP/PS1 mice in Hesperidin-treated arm showed a significant higher frequency of interactive behaviors as compared to control mice (control I=8.7±2.0, Hesperidin=22.7±5.7, *P*=0.04, Figure 12B). There was a trend towards significance of difference in the counts of interactive behaviors between Icariin-treated mice and their control littermates after 10-days treatment (control II=11.0±1.1, Icariin=15.7±1.3, DHM=11.7±1.7, *P*=0.06, Figure 12D). However, at any time point, the frequencies of independent behaviors in all polyphenol-treated mice were not statistically significantly different with control mice (Figure 13).

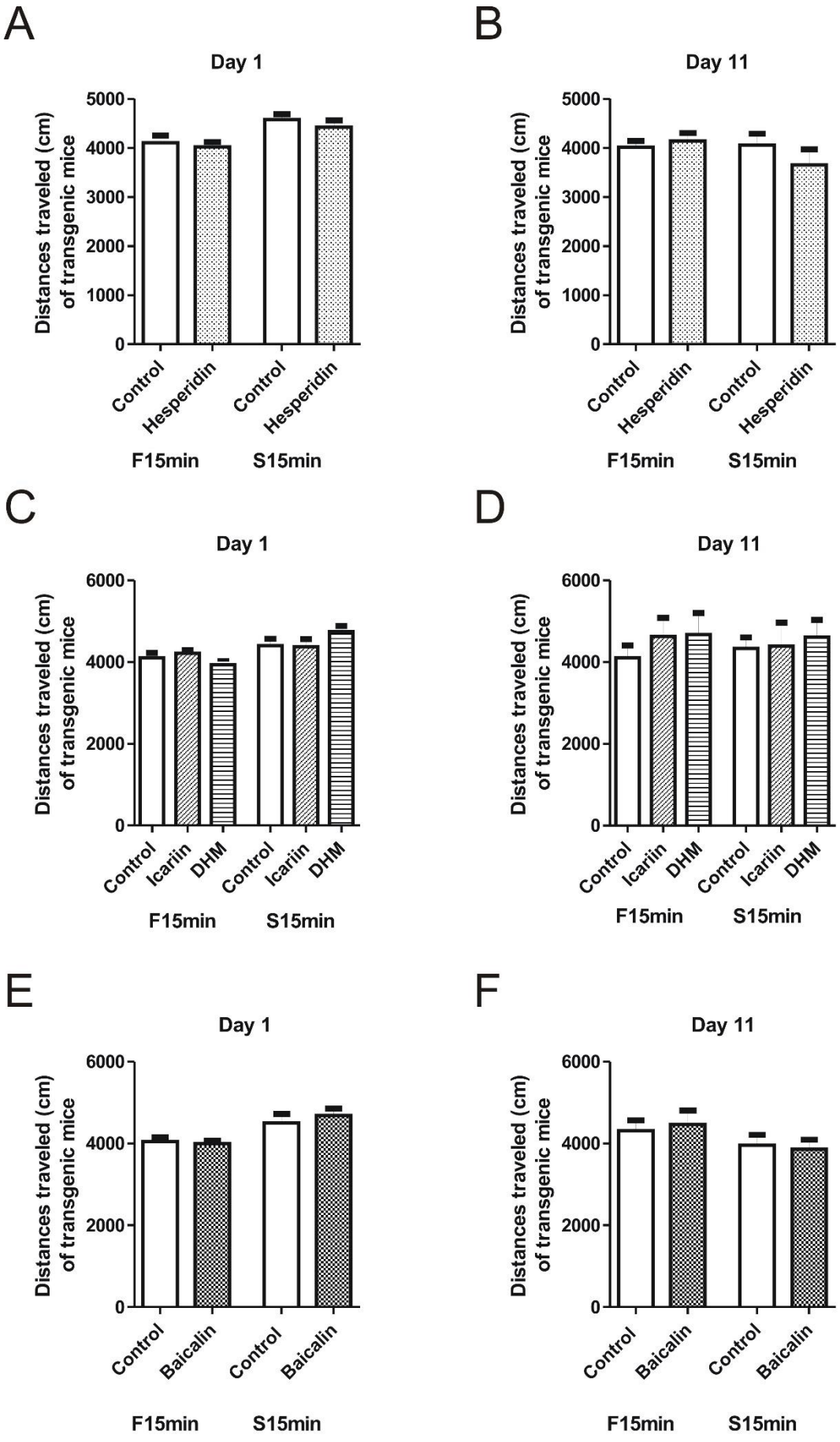

A
Day 1
Distances traveled (cm) of transgenic mice
5000
4000
3000
2000
1000
0
Control
Hesperidin
Control
Hesperidin
F15min
S15min
B
Day 11
Distances traveled (cm) of transgenic mice
5000
4000
3000
2000
1000
0
Control
Hesperidin
Control
Hesperidin
F15min
S15min
C
Day 1
Distances traveled (cm) of transgenic mice
6000
4000
2000
0
Control
Icariin
DHM
Control
Icariin
DHM
F15min
S15min
D
Day 11
Distances traveled (cm) of transgenic mice
6000
4000
2000
0
Control
Icariin
DHM
Control
Icariin
DHM
F15min
S15min
E
Day 1
Distances traveled (cm) of transgenic mice
6000
4000
2000
0
Control
Baicalin
Control
Baicalin
F15min
S15min
F
Day 11
Distances traveled (cm) of transgenic mice
6000
4000
2000
0
Control
Baicalin
Control
Baicalin
F15min
S15min

Figure 11 Distances traveled of resident mice in the resident-intruder assay. The distances traveled in Hesperidin- (A and B), Icariin- (C and D), DHM- (C and D) and Baicalin- (E and F) treated mice were not significantly changed compared to their respective control mice no matter prior to or after treatment.

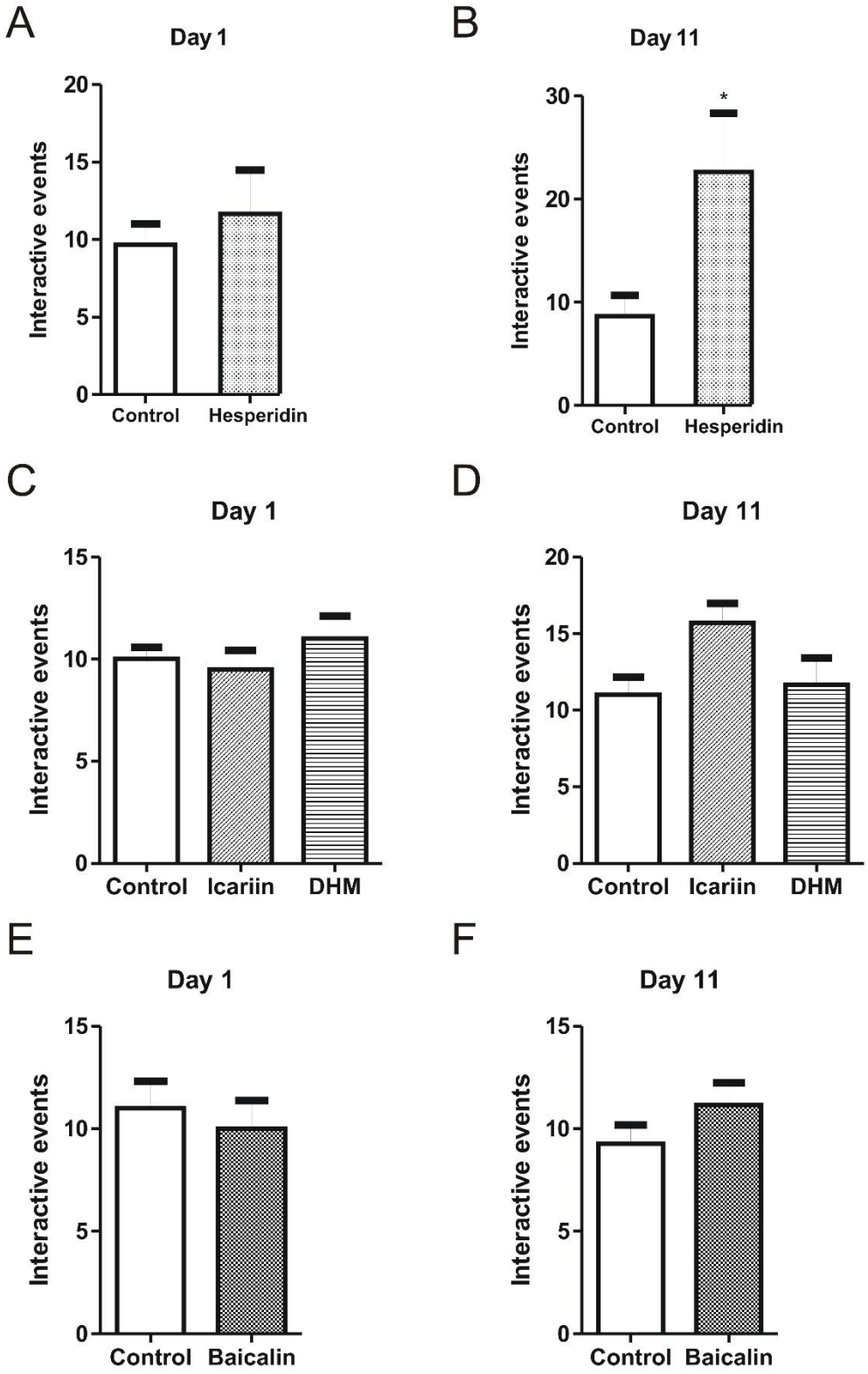


Figure 12 Numbers of interactive events for resident mice in the resident-intruder assay. (A) The difference in the number of interactive events between Hesperidin-treated and control mice was not statistically significant on Day 1. (B) After a 10-days treatment, the difference

in the number of interactive events between Hesperidin-treated and control group was obviously significant. (C-F) In the Icariin-, DHM- and Baicalin-treated group, the interactive event counts were not significant different with their respective control counterparts. $^{*}P<0.05$ compared to control group.

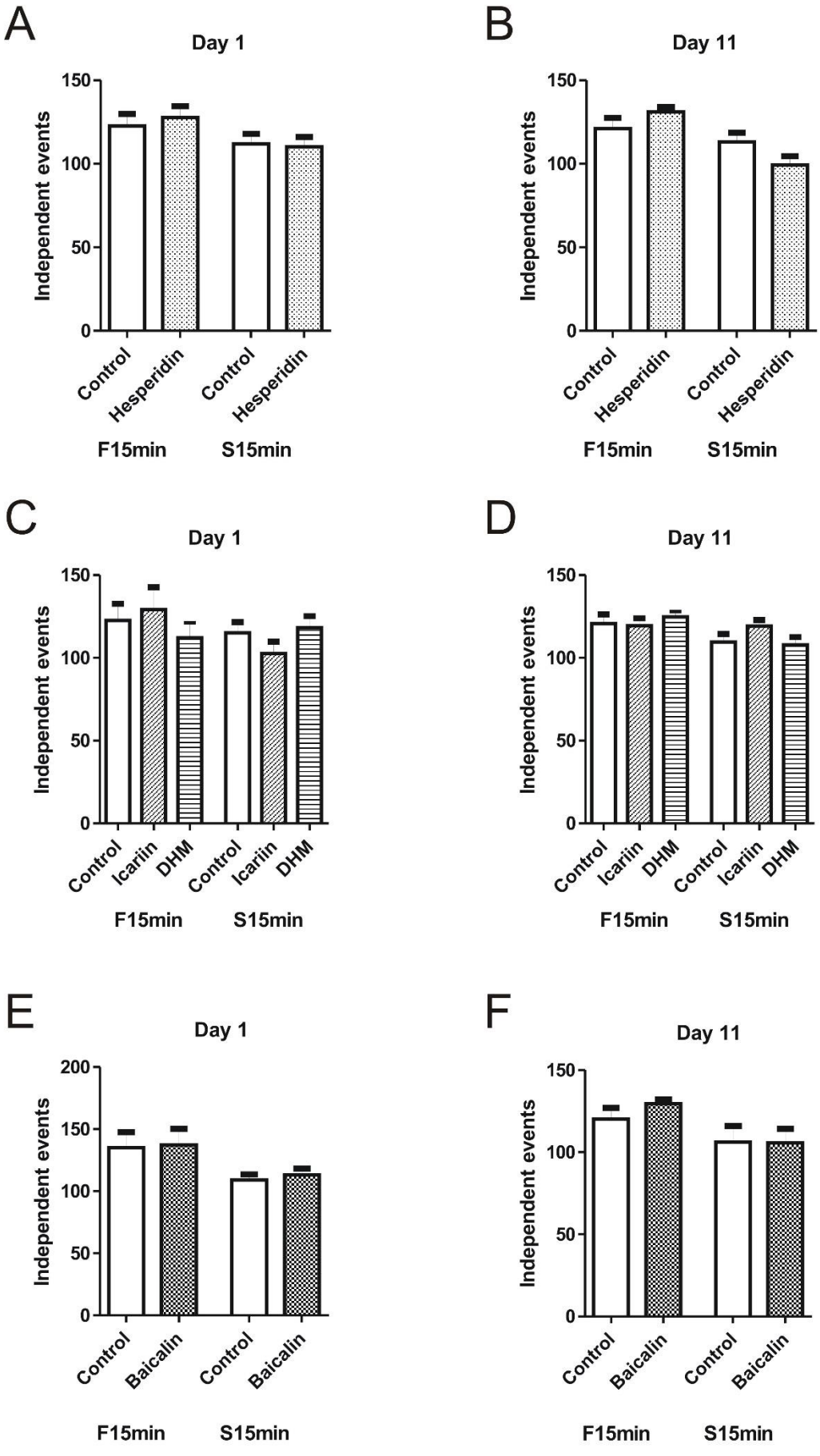

A
Day 1
Independent events
150
100
50
0
Control
Hesperidin
Control
Hesperidin
F15min
S15min
B
Day 11
Independent events
150
100
50
0
Control
Hesperidin
Control
Hesperidin
F15min
S15min
C
Day 1
Independent events
150
100
50
0
Control
Icariin
DHM
Control
Icariin
DHM
F15min
S15min
D
Day 11
Independent events
150
100
50
0
Control
Icariin
DHM
Control
Icariin
DHM
F15min
S15min
E
Day 1
Independent events
200
150
100
50
0
Control
Baicalin
Control
Baicalin
F15min
S15min
F
Day 11
Independent events
150
100
50
0
Control
Baicalin
Control
Baicalin
F15min
S15min

Figure 13 Numbers of independent events for resident mice in the resident-intruder assay. The numbers of independent events in Hesperidin- (A and B), Icariin- (C and D), DHM- (C and D) and Baicalin- (E and F) treated mice were all not significantly changed compared to their respective control mice at any time point.

3.3 Amelioration of AD-like pathology of APP/PS1 mice

3.3.1 Effects of four polyphenols on Aβ accumulation in brains of APP/PS mice

Amyloid plaques were distributed throughout the cortex of 5-months old transgenic APP/PS1 mice, some of them were small, dense core plaques and some were larger plaques with a dense core and a large halo of diffuse amyloid (Figure 14 A and C; Figure 15 A, C and E; Figure 16 A and C). In hippocampus, plaque density was distinctly lower (Figure 14 Band D; Figure 15 B, D and F; Figure 16 B and D).

We then evaluated the relative efficacies of Hesperidin, Icariin, DHM and Baicalin on plaque pathology in the APP/PS1 mouse during plaque deposition. Immunostaining for anti-Aβ revealed that there was a significant reduction in plaque numbers in the cortex of Hesperidin- and Icariin-treated mice, compared to their respective control mice (Experiment I: control I=157.8±4.2, Hesperidin=124.3±8.1, $P<0.01$, Figure 14E; Experiment II: control II=155.4±13.2, Icariin=95.0±12.6, DHM=139.5±10.3, $P<0.01$, Figure 15G; Experiment III: control III=164.2±8.8, Baicalin=150.0±10.2, $P=0.32$, Figure 16E). In the hippocampus, the difference in the plaque numbers between Icariin group and control group was statistically significant (Experiment I: control I=26.3±5.3, Hesperidin=16.7±3.8, $P=0.17$, Figure 14F; Experiment II: control II=21.6±3.4, Icariin=10.4±2.1, DHM=15.3±2.3, $P=0.03$, Figure 15H; Experiment III: control III=24.2±4.3, Baicalin=19.7±2.5, $P=0.38$, Figure 16F). Quantitative analysis of the amyloid burden, defined as the percent tissue area under examination occupied by Aβ plaques, was determined by MetaMorph software. After 10 days of Hesperidin administration, the Aβ IR areas were significantly reduced in the cortex (Experiment I: control I=0.7±0.1%, Hesperidin=0.4±0.0%, $P<0.01$, Figure 14G; Experiment II: control II=0.7±0.1%, Icariin=0.5±0.1%, DHM=0.6±0.1%, $P=0.17$, Figure 15I; Experiment III: control III=0.9±0.0%, Baicalin=0.7±0.1%, $P=0.08$, Figure 16G) and hippocampus (Experiment I: control I=0.5±0.1%, Hesperidin=0.3±0.1%, $P=0.03$, Figure 14H; Experiment II: control II=0.6±0.1%, Icariin=0.3±0.1%, DHM=0.5±0.2%,

P=0.12, Figure 15J; Experiment III: control III=0.7 ±0.1%, Baicalin=0.6 ±0.1%, *P*=0.37, Figure 16H) of Hesperidin-treated animals compared to control animals. It should be noticed that, no matter in the cortex or in the hippocampus of brain sections from DHM- and Baicalin-treated mice, the plaque numbers and IR areas were all not significant changed as compared to their respective controls.

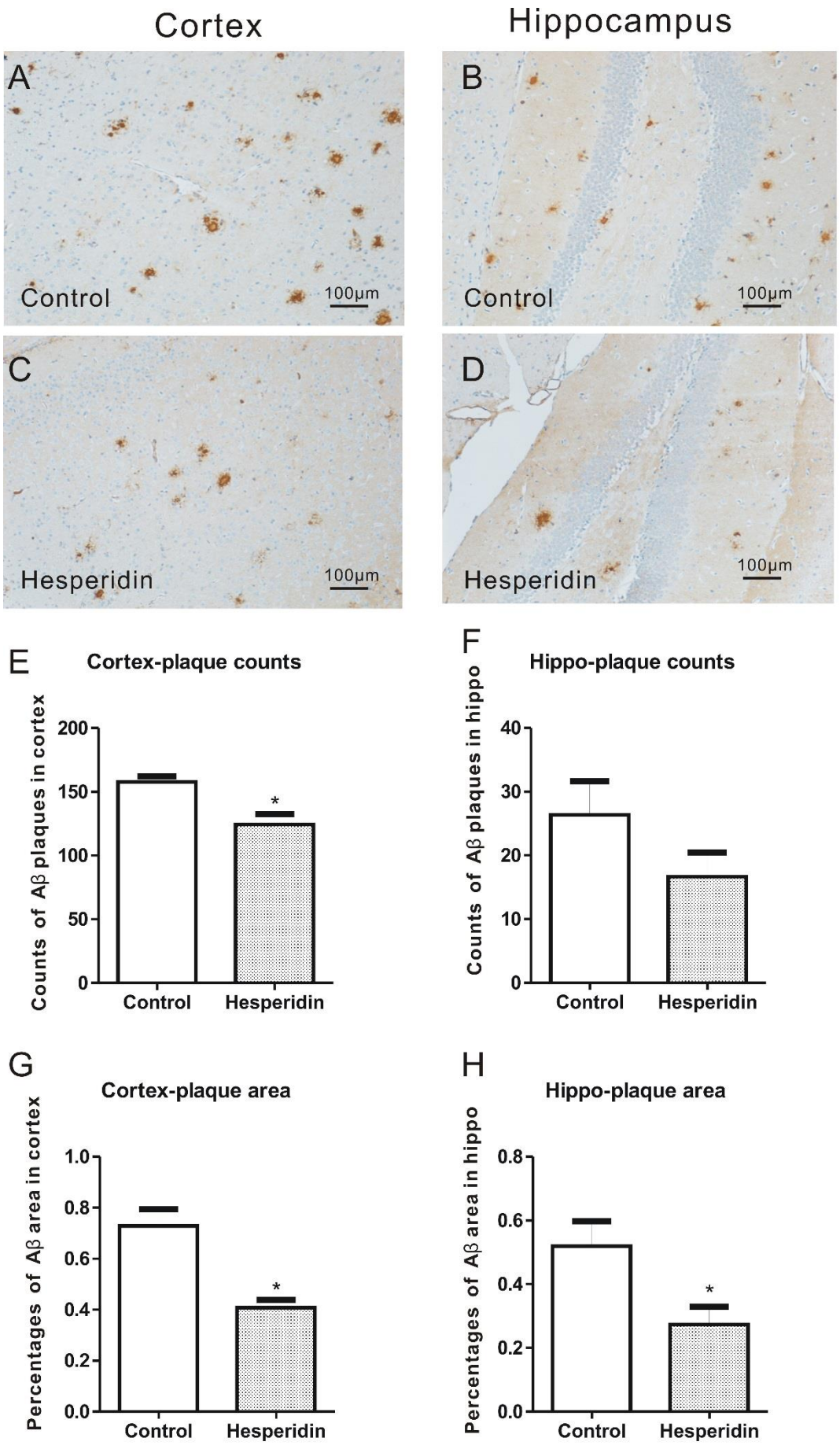

Cortex
Hippocampus
A
Control
100μm
B
Control
100μm
C
Hesperidin
100μm
D
Hesperidin
100μm
E
Cortex-plaque counts
Counts of Aβ plaques in cortex
200
150
100
50
0
*
Control
Hesperidin
F
Hippo-plaque counts
Counts of Aβ plaques in hippo
40
30
20
10
0
Control
Hesperidin
G
Cortex-plaque area
Percentages of Aβ area in cortex
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
Hesperidin
H
Hippo-plaque area
Percentages of Aβ area in hippo
0.8
0.6
0.4
0.2
0.0
*
Control
Hesperidin

Figure 14 Hesperidin reduced cerebral amyloidosis in transgenic APP/PS1 mice.

Brain coronal sections from APP/PS1 mice treated by Hesperidin or vehicle. (A-D) Representative images of Aβ immuno-reactivity showed the changes of amyloid plaques in the mouse brains following treatment with Hesperidin. In cortex and hippocampus of mice treated by Hesperidin (C and D), less and relatively small-size Aβ plaques were found, compared to control group (A and B). (E and F) The number of plaques in cortex of Hesperidin-treated mice was significantly reduced (E). Though the amyloid plaque count in hippocampus of mice treated by Hesperidin was lower as compared to the control group, the difference was not statistically significant (F). (G and H) Aβ immuno-reactive areas in cortex (G) and hippocampus (H) of Hesperidin-treated mice were obviously decreased. $^{*}P<0.05$ compared to control group. Hippo: Hippocampus.

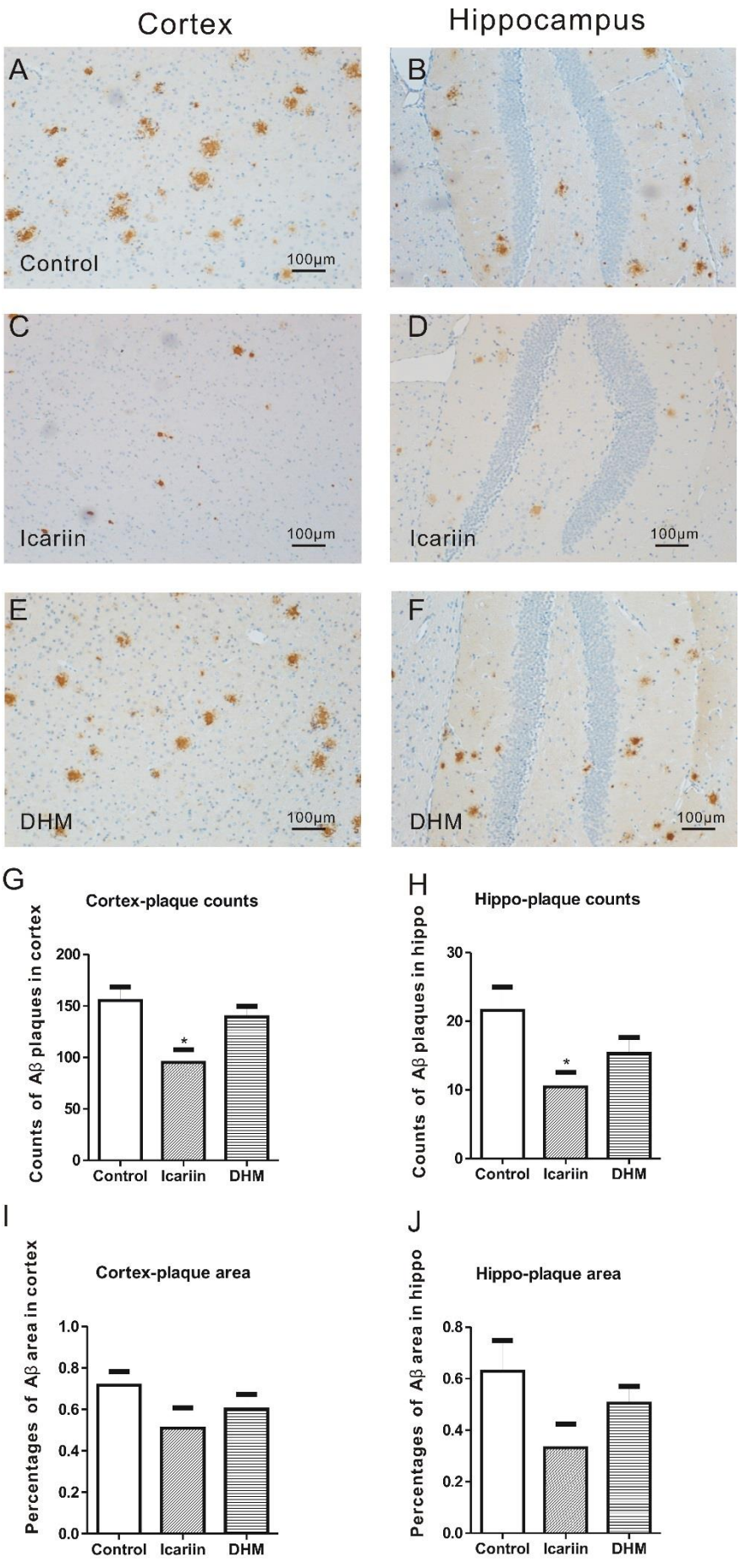

Cortex
Hippocampus
A
Control
100μm
B
C
Icariin
100μm
D
Icariin
100μm
E
DHM
100μm
F
DHM
100μm
G
Cortex-plaque counts
Counts of Aβ plaques in cortex
200
150
100
50
0
*
Control
Icariin
DHM
H
Hippo-plaque counts
Counts of Aβ plaques in hippo
30
20
10
0
*
Control
Icariin
DHM
I
Cortex-plaque area
Percentages of Aβ area in cortex
1.0
0.8
0.6
0.4
0.2
0.0
Control
Icariin
DHM
J
Hippo-plaque area
Percentages of Aβ area in hippo
0.8
0.6
0.4
0.2
0.0
Control
Icariin
DHM

Figure 15 Therapeutic effects of Icariin and DHM on cerebral amyloidosis of transgenic APP/PS1 mice. Brain coronal sections from APP/PS1 mice treated by Icariin, DHM or vehicle. (A-F) Representative images of Aβ immuno-reactivity showed changes of amyloid plaques in the mouse brains following treatment with Icariin and DHM. In cortex and hippocampus from mice treated by Icariin (C and D), less and relatively small-size Aβ plaques were found, compared to control groups (A and B). The numbers and morphology of Aβ plaques from the brains of DHM-treated mice were similar to control groups (E and F). (G and H) The numbers of amyloid plaques in the cortex (G) and hippocampus (H) of Icariin-treated mice were significantly lower than control mice, while the differences of plaque numbers in the brains between DHM-treated mice and control mice were not significant. (I and J) Percentages of Aβ IR area in the cortex and hippocampus from Icariin- and DHM- treated mice were not obviously changed compared to control mice. $^{*}P<0.05$ compared to control group. Hippo: Hippocampus.

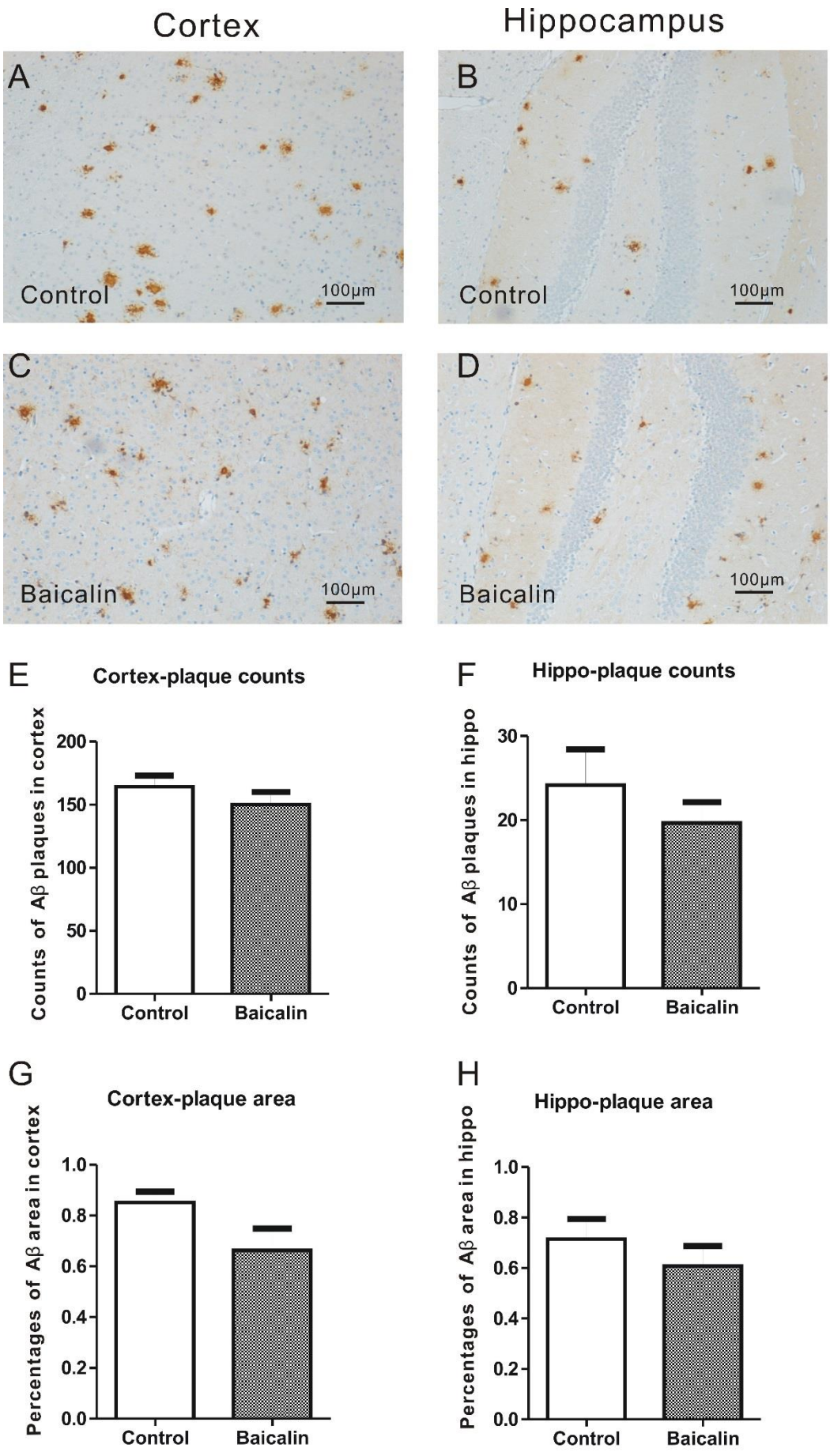

Cortex
Hippocampus
A
Control
100μm
B
Control
100μm
C
Baicalin
100μm
D
Baicalin
100μm
E
Cortex-plaque counts
Counts of Aβ plaques in cortex
200
150
100
50
0
Control
Baicalin
F
Hippo-plaque counts
Counts of Aβ plaques in hippo
30
20
10
0
Control
Baicalin
G
Cortex-plaque area
Percentages of Aβ area in cortex
1.0
0.8
0.6
0.4
0.2
0.0
Control
Baicalin
H
Hippo-plaque area
Percentages of Aβ area in hippo
1.0
0.8
0.6
0.4
0.2
0.0
Control
Baicalin

Figure 16 Therapeutic effect of Baicalin on cerebral amyloidosis of transgenic APP/PS1 mice. Brain coronal sections from APP/PS1 mice treated by Baicalin or vehicle. (A-D) Representative images of Aβ immuno-reactivity showed the changes of amyloid plaques in the mouse brains following treatment with Baicalin. Both in cortex and hippocampus from mice treated by Baicalin (C and D), the numbers and morphology of amyloid plaques were similar to control mice (A and B). (E-H) Both in cortex and hippocampus, the differences in the numbers and IR areas of amyloid plaques between Baicalin-treated and control mice were not statistically significant. Hippo: Hippocampus.

3.3.2 Effects of four polyphenols on activation of microglia and astrocytes in brains of APP/PS mice

Apart from Aβ accumulation, activation of microglia and astrocytes is also supposed to be one of the characteristic features of AD; they could produce pro-inflammatory cytokines and generate Aβ on activation.

Activation of microglia was characterized by morphological changes from ramified (quiescent) morphology to amoeboid (activated) morphology, and increased microglial cell number. From the representative pictures shown in Figure 17-19, the amoeboid Iba-1 positive microglia distributed throughout the cortex and hippocampus of 5-months old APP/PS1 mice. To determine the number of microglial cells in the brains of APP/PS1 mice, we performed stereological cell counts. In sum, Iba-1 reactive cell numbers were reduced in the cortex (Experiment I: control I=88.2±4.8, Hesperidin=36.0±3.9, $P<0.01$, Figure 17E; Experiment II: control II=103.3±9.4, Icariin=44.2±4.9, DHM=92.7±5.1, $P<0.01$, Figure 18G; Experiment III: control III=86.8±4.6, Baicalin=84.8±5.1, $P=0.78$, Figure 19E) and hippocampus (Experiment I: control I=36.3±3.6, Hesperidin=16.0±1.0, $P<0.01$, Figure 17F; Experiment II: control II=30.1±1.8, Icariin=17.6±1.4, DHM=27.3±1.5, $P<0.01$, Figure 18H; Experiment III: control III=30.7±2.5, Baicalin=26.0±1.7, $P=0.15$, Figure 19F) of Hesperidin- and Icariin-treated mice compared to their respective control mice. Significant decreases in Iba-1 IR areas were also observed in the cortex (Experiment I: control I=0.38±0.02%, Hesperidin=0.24±0.01%, $P<0.01$, Figure 17G; Experiment II: control II=0.35±0.06%, Icariin=0.21±0.04%, DHM=0.37±0.02%, $P=0.04$, Figure 18I; Experiment III: control III=0.53±0.06%, Baicalin=0.40±0.04%, $P=0.10$, Figure 19G) and hippocampus (Experiment I: control I=0.30±0.03%, Hesperidin=0.19±0.03%, $P=0.02$, Figure 17H; Experiment II: control II=0.29±0.04%, Icariin=0.16±0.04%, DHM=0.30±0.02%, $P=0.03$, Figure 18J; Experiment III: control III=0.32±0.02%, Baicalin=0.27±0.02%, $P=0.10$, Figure 19H) of the mice treated by Hesperidin and

Icariin, compared to control mice. Both in the cortex and hippocampus, the Iba-1 reactive cell numbers and IR areas from DHM- and Baicalin-treated mice were not significant different from their respective controls.

Astrocyte activation also resulted in morphological changes, including stellate shape with the shortening and thickening of processes, and increased proliferation (Figure 20-22). After the 10-days treatment, the GFAP positive cell numbers and IR areas in the brains of mice treated by all these polyphonic compounds were not significant changed, compared to their respective control mice (Figure 20-22).

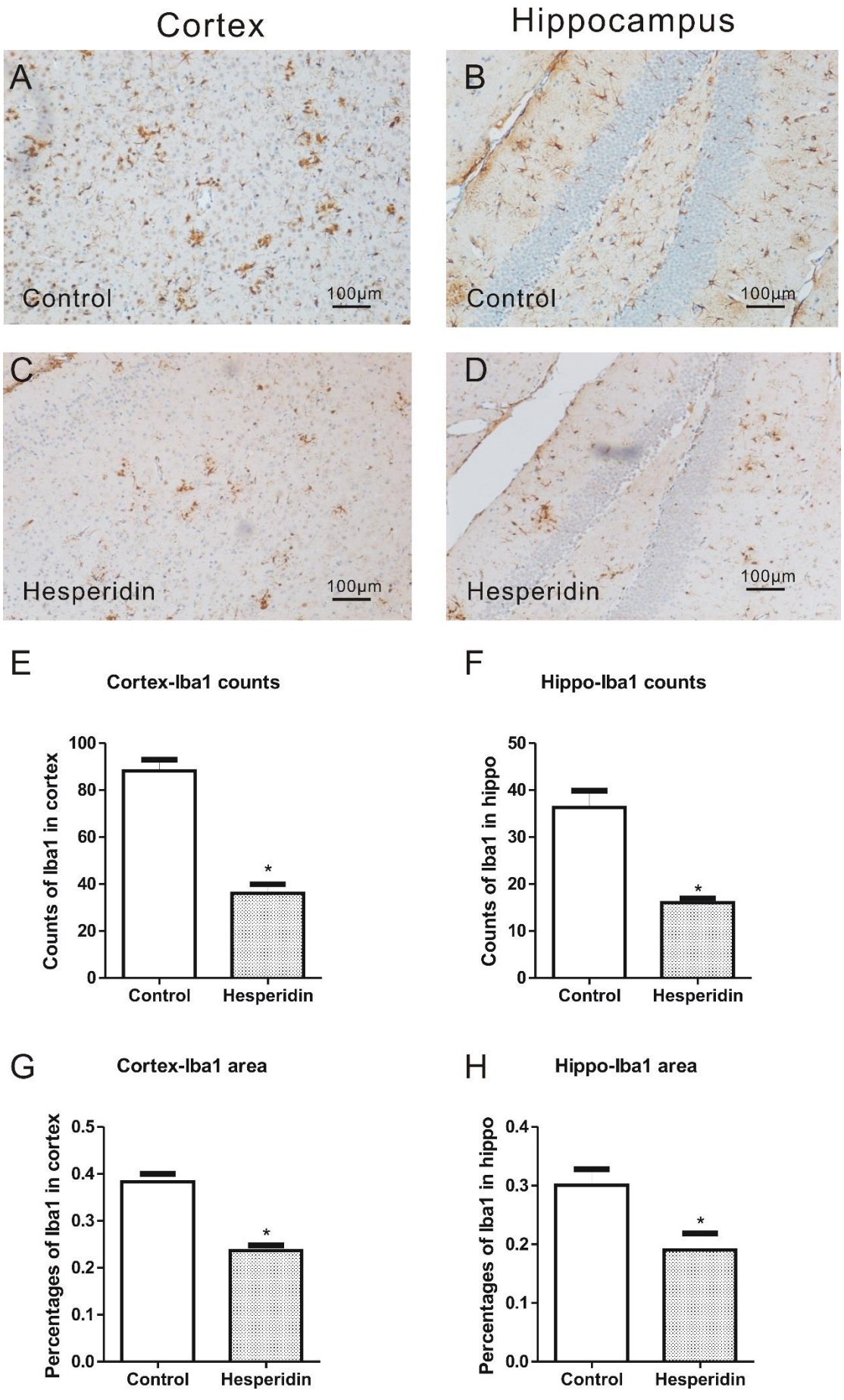
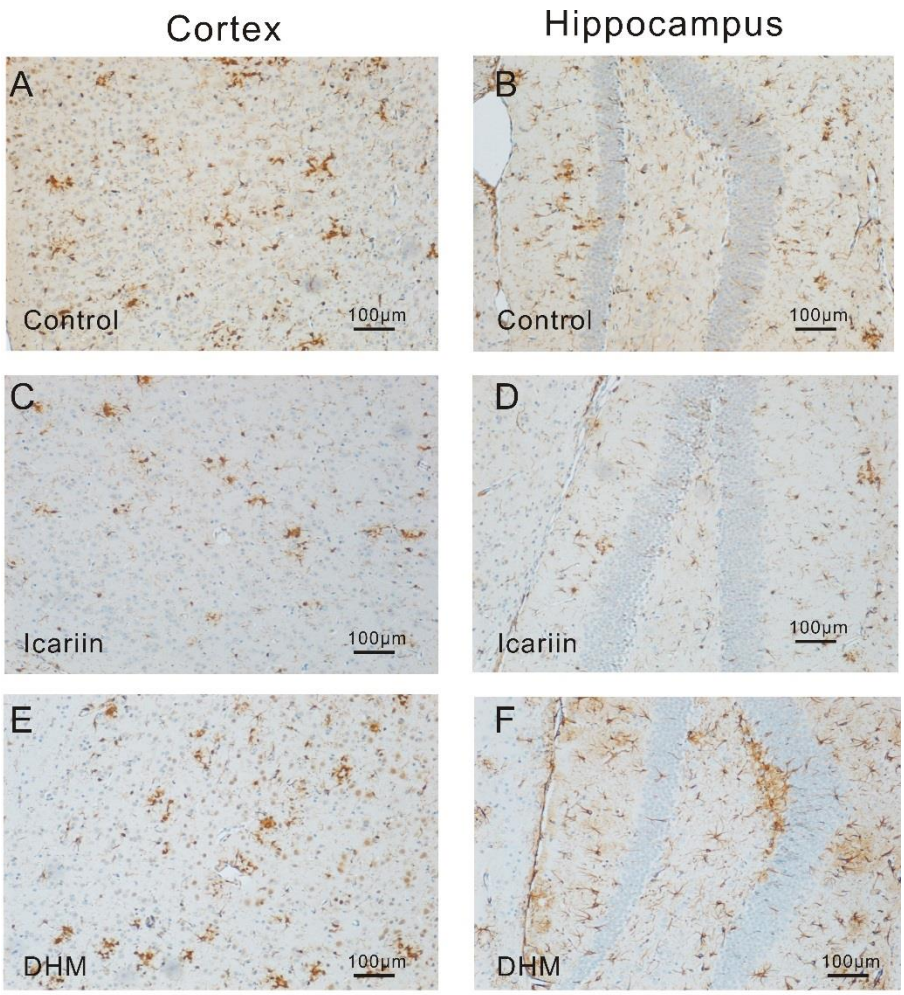
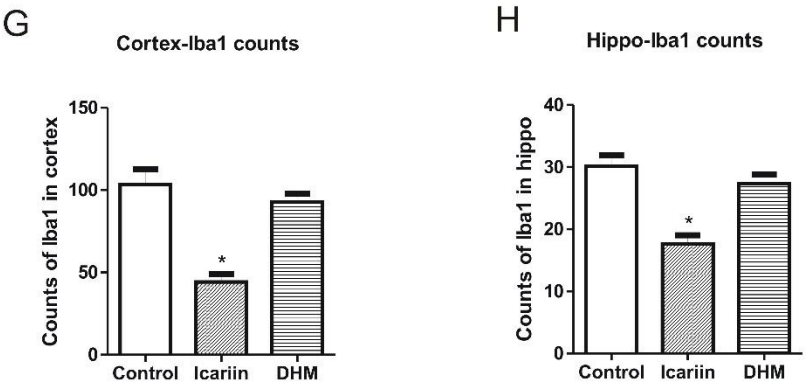

Cortex
Hippocampus
A
Control
100μm
B
Control
100μm
C
Hesperidin
100μm
D
Hesperidin
100μm
E
Cortex-Iba1 counts
Counts of Iba1 in cortex
100
80
60
40
20
0
*
Control
Hesperidin
F
Hippo-Iba1 counts
Counts of Iba1 in hippo
50
40
30
20
10
0
*
Control
Hesperidin
G
Cortex-Iba1 area
Percentages of Iba1 in cortex
0.5
0.4
0.3
0.2
0.1
0.0
*
Control
Hesperidin
H
Hippo-Iba1 area
Percentages of Iba1 in hippo
0.4
0.3
0.2
0.1
0.0
*
Control
Hesperidin

Figure 17 Hesperidin ameliorates microglial activation in transgenic APP/PS1 mice. Brain coronal sections from APP/PS1 mice treated by Hesperidin or vehicle. (A-D) Representative images of Iba-1 immuno-reactivity showed the changes of microglial cells in the cortex and hippocampus following treatment with Hesperidin. Both in cortex and hippocampus from mice treated by Hesperidin (C and D), less and relatively small IR area of Iba-1 positive cells were found, compared to control mice (A and B). (E-H) Both in cortex and hippocampus, the numbers and IR areas of Iba-1 positive cells in the mice treated by Hesperidin were significant reduced, compared to control mice. $^{*}P<0.05$ compared to control group. Hippo: Hippocampus.


Cortex
Hippocampus
A
Control
100μm
B
Control
100μm
C
Icariin
100μm
D
Icariin
100μm
E
DHM
100μm
F
DHM
100μm


G
Cortex-Iba1 counts
Counts of Iba1 in cortex
150
100
50
0
*
Control
Icariin
DHM
H
Hippo-Iba1 counts
Counts of Iba1 in hippo
40
30
20
10
0
*
Control
Icariin
DHM

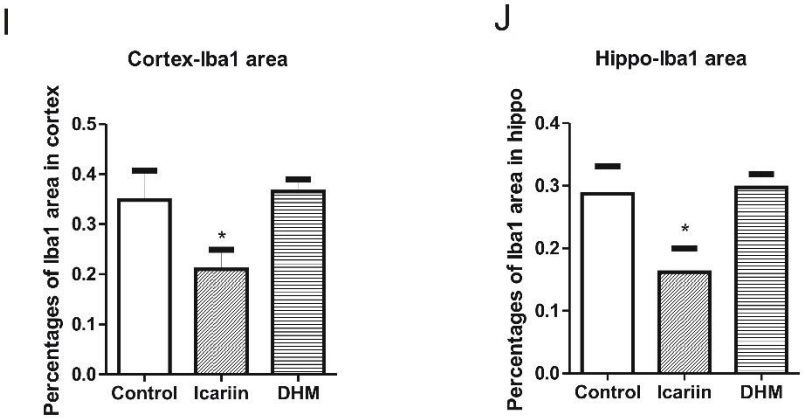

I
Cortex-Iba1 area
Percentages of Iba1 area in cortex
0.5
0.4
0.3
0.2
0.1
0.0
*
Control
Icariin
DHM
J
Hippo-Iba1 area
Percentages of Iba1 area in hippo
0.4
0.3
0.2
0.1
0.0
*
Control
Icariin
DHM

Figure 18 Therapeutic effect of Icariin and DHM on microglia activation in transgenic APP/PS1 mice. Brain coronal sections from APP/PS1 mice treated by Icariin, DHM or vehicle. (A-F) Representative images of Iba-1 immuno-reactivity showed the changes of microglial cells in the cortex and hippocampus following treatment with Icariin or DHM. In the cortex and hippocampus from mice treated by Icariin (C and D), less and relatively small IR area of Iba-1 positive cells were found, compared to the control group (A and B). The numbers and morphology of Iba-1 positive cells from the brains of DHM-treated mice were similar to control group (E and F). (G-J) Both in cortex and hippocampus, the numbers and IR areas of Iba-1 positive cells in the mice treated by Icariin were significant reduced compared to control mice. $^{*}P<0.05$ compared to control group. Hippo: Hippocampus.

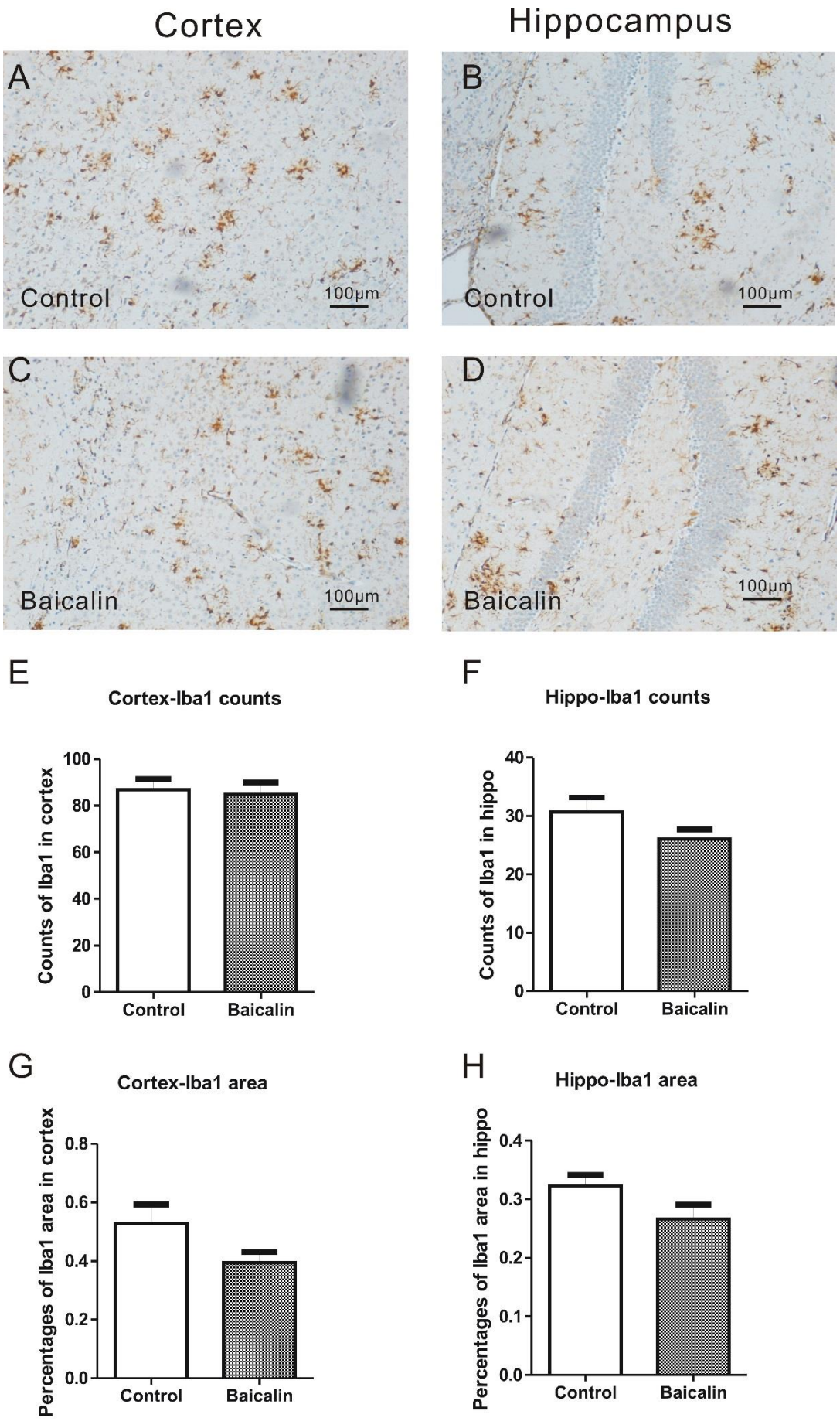

Cortex
Hippocampus
A
Control
100μm
B
Control
100μm
C
Baicalin
100μm
D
Baicalin
100μm
E
Cortex-Iba1 counts
Counts of Iba1 in cortex
100
80
60
40
20
0
Control
Baicalin
F
Hippo-Iba1 counts
Counts of Iba1 in hippo
40
30
20
10
0
Control
Baicalin
G
Cortex-Iba1 area
Percentages of Iba1 area in cortex
0.8
0.6
0.4
0.2
0.0
Control
Baicalin
H
Hippo-Iba1 area
Percentages of Iba1 area in hippo
0.4
0.3
0.2
0.1
0.0
Control
Baicalin

Figure 19 Therapeutic effect of Baicalin on microglia activation in transgenic APP/PS1 mice. Brain coronal sections from APP/PS1 mice treated by Baicalin or vehicle. (A-D) Representative images of Iba-1 immuno-reactivity of microglial cells in cortex and hippocampus following treatment with Baicalin. In cortex and hippocampus from mice treated by Baicalin (C and D), the numbers and morphology of Iba-1 positive cells were similar to control mice (A and B). (E-H) The differences in the numbers and IR areas of Iba-1 positive cells between Baicalin-treated and control mice were not statistically significant. Hippo: Hippocampus.

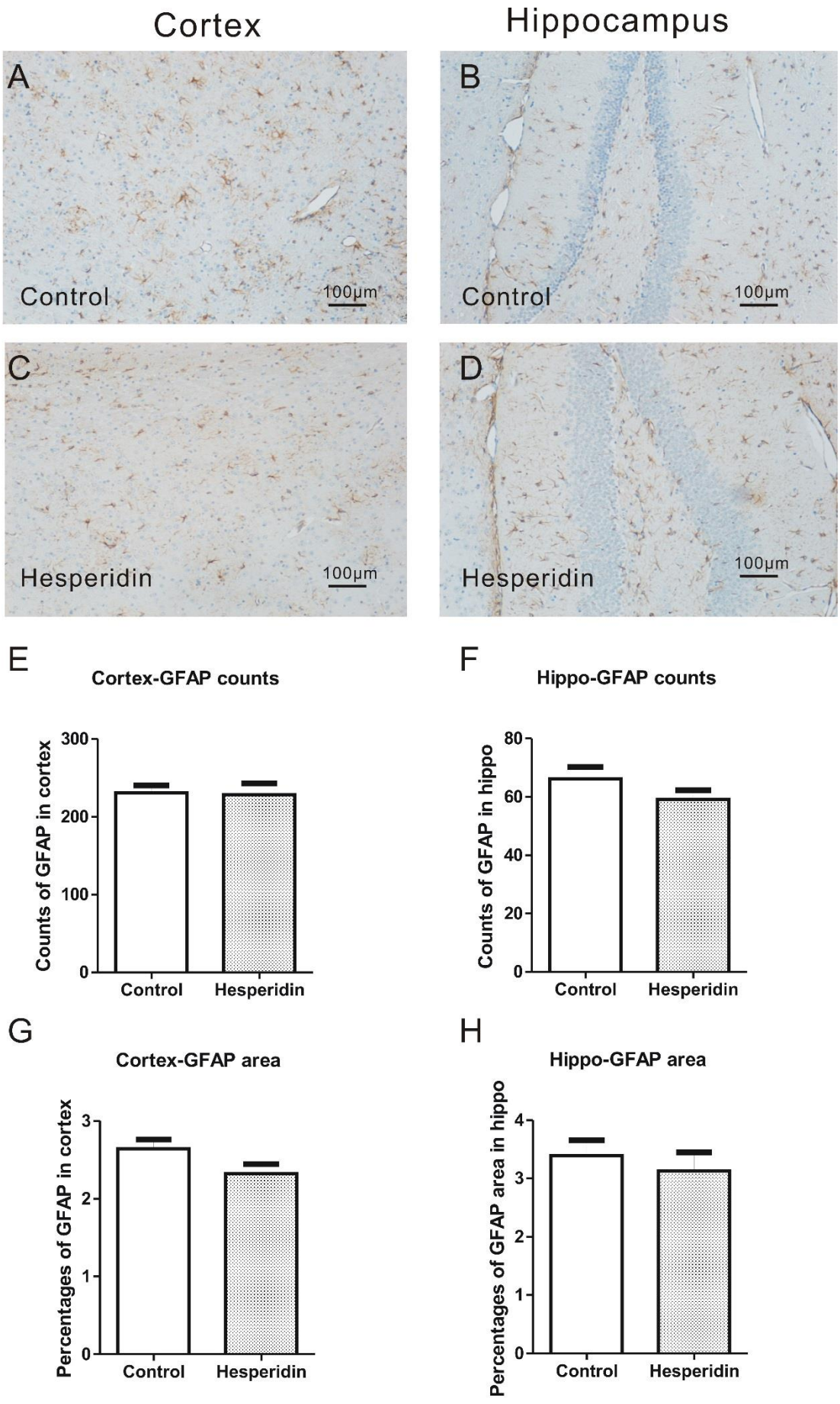
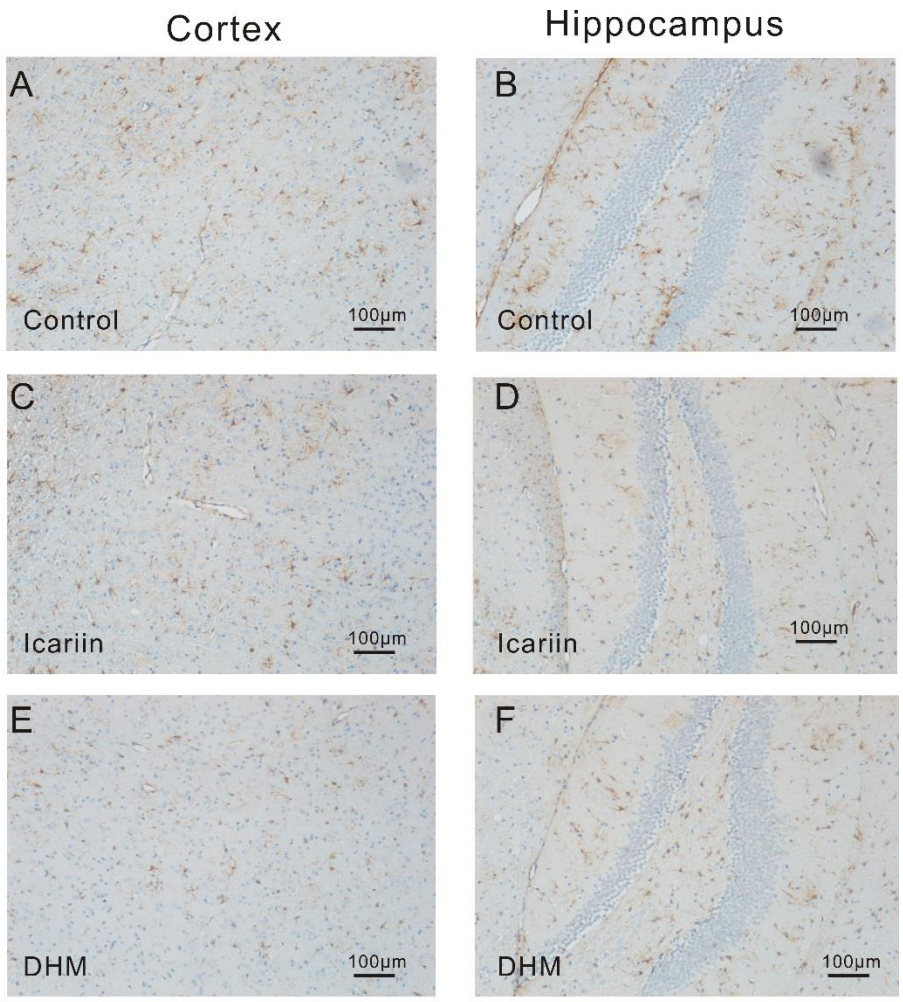
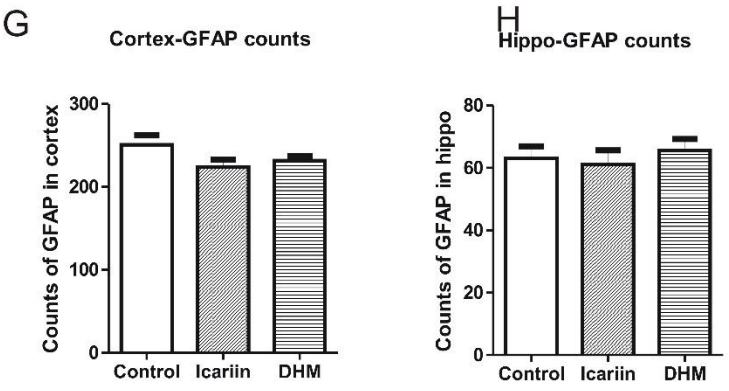

Cortex
Hippocampus
A
Control
100μm
B
Control
100μm
C
Hesperidin
100μm
D
Hesperidin
100μm
E
Cortex-GFAP counts
Counts of GFAP in cortex
300
200
100
0
Control
Hesperidin
F
Hippo-GFAP counts
Counts of GFAP in hippo
80
60
40
20
0
Control
Hesperidin
G
Cortex-GFAP area
Percentages of GFAP in cortex
3
2
1
0
Control
Hesperidin
H
Hippo-GFAP area
Percentages of GFAP area in hippo
4
3
2
1
0
Control
Hesperidin

Figure 20 Therapeutic effect of Hesperidin on the activation of astrocyte in transgenic APP/PS1 mice. Brain coronal sections from APP/PS1 mice treated by Hesperidin or vehicle. (A-D) Representative images of GFAP immuno-reactivity showed the changes of astrocytes in the cortex and hippocampus following treatment with Hesperidin. (E-H) The differences in the numbers and IR areas of GFAP positive cells between Hesperidin-treated and control mice were not statistically significant. Hippo: Hippocampus.


Cortex
Hippocampus
A
Control
100μm
B
Control
100μm
C
Icariin
100μm
D
Icariin
100μm
E
DHM
100μm
F
DHM
100μm


G
Cortex-GFAP counts
Counts of GFAP in cortex
300
200
100
0
Control
Icariin
DHM
H
Hippo-GFAP counts
Counts of GFAP in hippo
80
60
40
20
0
Control
Icariin
DHM

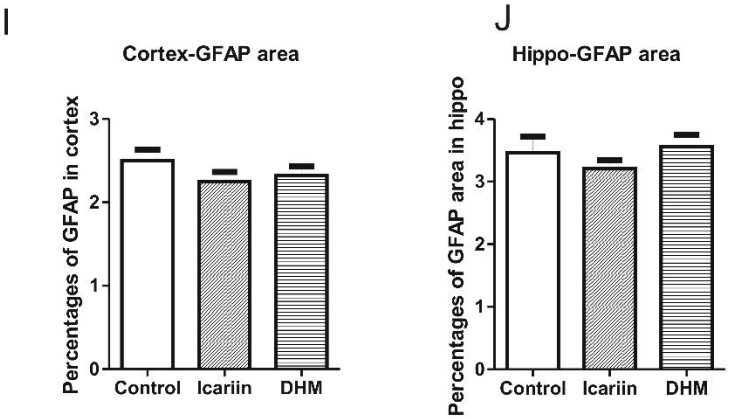

I
Cortex-GFAP area
Percentages of GFAP in cortex
3
2
1
0
Control
Icariin
DHM
J
Hippo-GFAP area
Percentages of GFAP area in hippo
4
3
2
1
0
Control
Icariin
DHM

Figure 21 Therapeutic effects of Icariin and DHM on the activation of astrocyte in transgenic APP/PS1 mice. Brain coronal sections from APP/PS1 mice treated by Icariin, DHM or vehicle. (A-F) Representative images of GFAP immuno-reactivity of astrocytes in the cortex and hippocampus following treatment with Icariin or DHM. (G-J) The differences in the numbers and IR areas of GFAP positive cells among control, Icariin- and DHM-treated mice were not statistically significant. Hippo: Hippocampus.

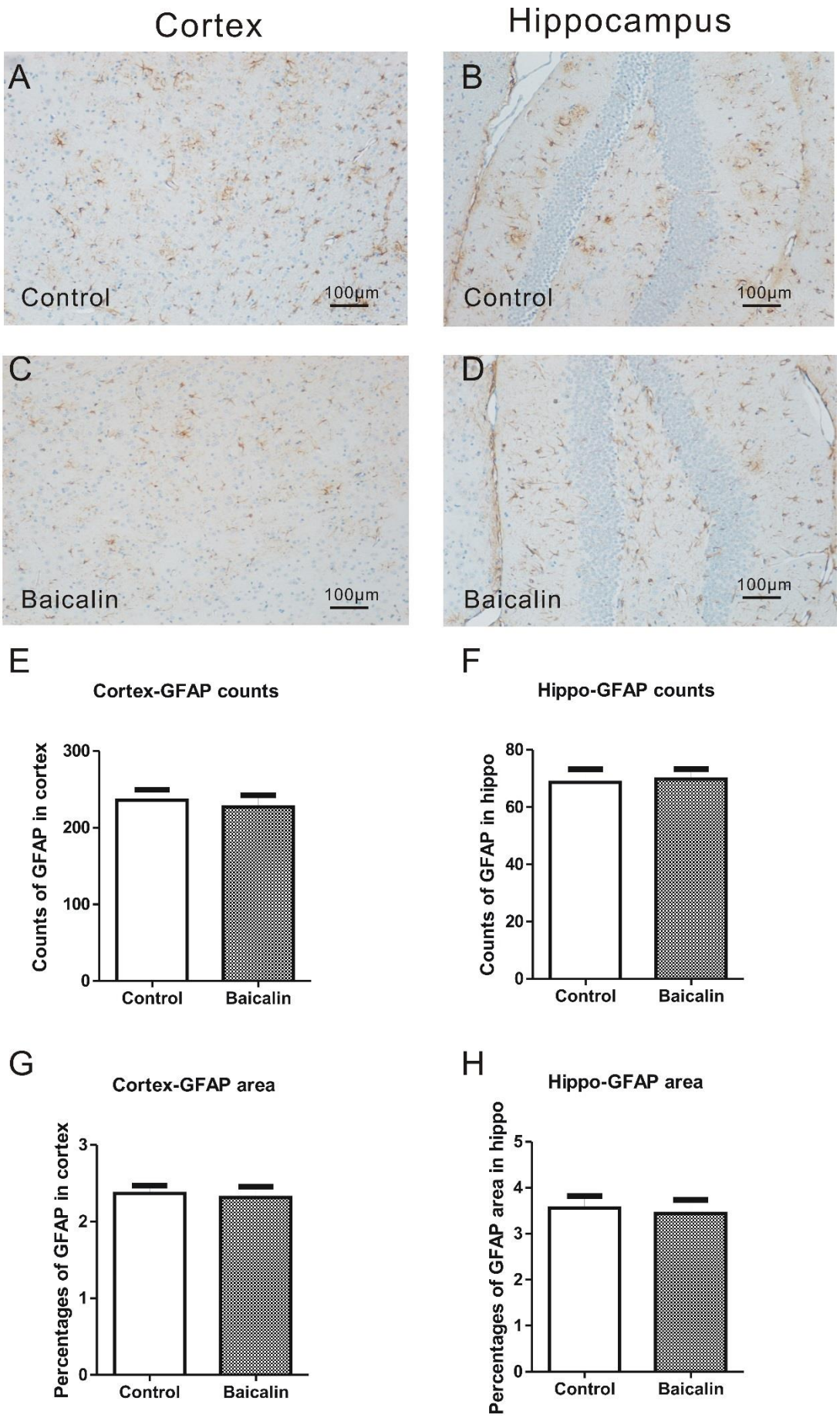

Cortex
Hippocampus
A
Control
100μm
B
Control
100μm
C
Baicalin
100μm
D
Baicalin
100μm
E
Cortex-GFAP counts
Counts of GFAP in cortex
300
200
100
0
Control
Baicalin
F
Hippo-GFAP counts
Counts of GFAP in hippo
80
60
40
20
0
Control
Baicalin
G
Cortex-GFAP area
Percentages of GFAP in cortex
3
2
1
0
Control
Baicalin
H
Hippo-GFAP area
Percentages of GFAP area in hippo
5
4
3
2
1
0
Control
Baicalin

Figure 22 Therapeutic effect of Baicalin on the activation of astrocyte in transgenic APP/PS1 mice. Brain coronal sections from APP/PS1 mice treated by Baicalin or vehicle. (A-D) Representative images of GFAP immuno-reactivity of astrocytes in cortex and hippocampus following treatment with Baicalin. (E-H) The differences in the numbers and IR areas of GFAP positive cells between Baicalin-treated mice and control mice were not statistically significant. Hippo: Hippocampus.

Chapter IV: Discussion

Our results showed that Hesperidin and Icariin treatment, of a relatively short term, restored impairment in nesting ability and in social interaction of APP/PS1 transgenic mice at 5 months of age. The following histological assay indicated that treatment with Hesperidin and Icariin significantly ameliorated accumulation of Aβ deposits, and reduced microglial activation in both cortex and hippocampus.

In consideration of the large amount of social and financial costs of neurodegeneration, considerable effort has been devoted to combating these devastating diseases. Over 140 compounds and treatment strategies have been employed in transgenic AD mouse models from 2001 to 2011 (summarized in our review, Li et al., 2013a). However, until now, no effective clinical drugs are available. A growing number of drugs were abandoned by pharmaceuticals companies, primarily because of efficacy and/or toxicity issues in preclinical rodent models or clinical trials for treatment of AD (Mori et al., 2013; Palmer, 2011).

Currently, use of many traditional herbal remedies is becoming more and more prevalent, since they are suggested to delay cerebral degeneration with rare adverse effects. While the application of herbal drugs is disputable, in Europe, Ginkgo biloba leaf extract is regarded as one of the most popular therapies for the treatment of AD (Garcia-Alloza et al., 2010). Huperzine A and galantamine are originated from traditional Chinese herbs and have been employed in clinics for treating mild to moderate AD (Takata et al., 2010; Wang et al., 2006b). In the long history of development of TCM, a large number of herbal medicines have been described and used to treat dementia in China and other East Asian countries. *The Complete Work of Jingyue,* which was published in 1624, described the earliest known herbal therapeutic strategy for dementia in the world (Tian et al., 2010). Recently, thanks to the development of spectroscopic and chromatographic techniques, an increasing number of agents have been isolated from these herbals and the evaluation of their efficacies in preclinical models of AD has been analyzed both *in vitro* and *in vivo* (Gao et al., 2013).

Natural polyphenols are a large group of phytochemical substances that are composed of aromatic rings and one or more phenolic rings. They may interact with the aromatic residue present in the amyloidogenic proteins and then inhibit the self-assembly process resulting into amyloid fibril formation (Porat et al., 2006). Hesperidin, Icariin, DHM and Baicalin, which are among the most abundant phenolic compounds, are all produced by and prepared from TCM herbs. These compounds have pleiotropic biological properties, including anti-oxidant and anti-inflammatory activities. Moreover, all of them exert little adverse effect, have low or no cytotoxicity (Shen et al., 2012; Wang et al., 2013b; Xue et al., 2012; Yang et al., 2012d), and cross the BBB (Chen et al., 2007; Guo et al., 2010; Li and Wang, 2008; Youdim et al., 2003). Given the biological activity profile, we hypothesized that the treatment with these four polyphenols may slow the progression of AD-like pathology and behavioral deficits, if administrated early. Therefore, transgenic APP/PS1 mice were treated daily by 100 mg/kg of the polyphenols by gavage. This therapeutic strategy delivers agents more precisely in comparison to ad libitum access to drinking water or chow (Mori et al., 2013).

Function deficits are common neurological sequelae in neurodegenerative diseases and their animal models (Chen et al., 2008). As memory and orientation impairment would not be observed until at 8 months of age in transgenic APP/PS1 mice, deficits in non-mnemonic behaviors, which also are debilitating features of AD and can be observed already during early AD development, were chosen for analysis of effects of Hesperidin, Icariin, DHM and Baicalin. Certain species-typical rodent behaviors, such as nest construction and social interactive behavior, are considered rodent equivalents of the non-cognitive behavior that deteriorates in AD.

Nesting is a common affiliative, social behavior in mice, and has been reported to simulate activities of daily living in AD transgenic mice (Torres-Lista and Gimenez-Llort, 2013). One of our previous studies proved impaired nesting ability of transgenic

APP/PS1 mice as compared with naive mice (Zhang and Schluesener, 2013). It has been suggested, that the hippocampus and the prefrontal cortex damage in mice results in reduced nesting material consumption and nest quality, indicating that the impairment of nesting behavior in APP/PS1 mice might be caused by toxic injury by Aβ, accompanying neuro-inflammation (Wesson and Wilson, 2011b).

After 10-days treatment, the nest built by Hesperidin- and Icariin-treated mice were of improved quality, indicating that Hesperidin and Icariin might exert beneficial effects on AD transgenic mice within such a short-term treatment.

Patients with AD express social withdrawal, sometimes even associated with dysphoria and depression (Chung and Cummings, 2000; Frisoni et al., 1999). Brain regions responsible for social memory are the perirhinal cortex and hippocampus (Brown and Aggleton, 2001). Both regions are compromised with AD. Thus, social communication of APP/PS1 mice might be impaired by pathological changes in these brain regions as well, including Aβ deposition and neuro-inflammation. In support of this view, a previous study revealed that APPswe/PS1 transgenic mice, a similar AD animal model, were less socially active with stimulus mice, than wild-type mice (Filali et al., 2011). Impaired social interaction was also reported in our transgenic APP/PS1 mouse model previously (Zhang et al., 2013c). The differences in distances travelled and independent behaviour numbers between the treatment groups and their respective controls were not statistically significant, indicating that the motor function of these mice were normal. One this basis, the impaired social interaction of mice was significantly improved by Hesperidin treatment, especially at this relatively young age. Considering the relatively short term of the treatment, reduced Aβ deposition and especially attenuated neuro-inflammatory reaction may contribute to the improved affiliative nesting behavior and social interaction.

Several *in vitro* and *in vivo* studies have demonstrated that Hesperidin and Icariin effectively attenuated inflammatory reaction and significantly decreased the levels of

pro-inflammatory cytokines/molecules (Choi et al., 2007; Emim et al., 1994; Wang et al., 2013a; Xu et al., 2010; Zhang et al., 2012a). Given the potential ability of crossing the BBB, Hesperidin and Icariin might further have inhibitory effects on neuro-inflammation. Our histological results showed that Hesperidin and Icariin significantly reduced the number and IR area of Iba-1 positive microglia cells in both cortex and hippocampus of APP/PS1 transgenic mice. These results suggested an inhibitory effect of Hesperidin and Icariin on neuro-inflammation in this transgenic mouse model, which may contribute to ameliorated pathology and improved behavior.

An important role of neuro-inflammation involved in AD pathology has been reported in rodent models and humans (Martin-Moreno et al., 2012a). Microglial cells are the most important immune effector cells in the brain. They act the role of cerebral resident macrophages, which could maintain brain homeostasis and protect brains from insults and infections (Blasko et al., 2004). Microglial activation is one hallmark of neuro-inflammation. Amyloid peptides and their precursor proteins are potent glial activators (Barger and Harmon, 1997; Rangasamy et al.), and cause microglia to produce cytokines like IL-1β and TNF−α. The inflammatory reaction is then mediated by these pro-inflammatory cytokines and would create a chronic, self-sustaining inflammatory interaction between activated microglia, stressed neurons and Aβ plaques (Rubio-Perez and Morillas-Ruiz, 2012). In an in vitro assay, activation of microglia from AD patients and non-demented controls with Aβ peptide released pro-inflammatory cytokines (TNF-α and IL-1β) in a dose-response pattern (Lue et al., 2001; Rogers and Lue, 2001). A number of autopsy diagnoses also showed the presence of these microglial cytokine in close proximity to AD lesions (Dickson, 1997; Griffin et al., 1995). Microglial activation and expression of these inflammatory molecules/cytokines are directly involved in the development of neuro-inflammation and in neurodegenerative pathology. The levels of these cytokines/molecules were even reported to correlate with amyloid load in a similar transgenic mouse model of AD (Patel et al., 2005). Inflammatory reaction and inflammatory cytokines/molecules were reported to be

directly associated with deficits in behaviors and cognitive function (Ownby, 2010). A large amount of preclinical and clinical studies have reported that Hesperidin and Icariin inhibited the mRNA expression of pro-inflammatory cytokines (e.g. IL-1β, IL-6, TNF-α and iNOS) (Chen et al., 2010b; Guo et al., 2010; Rizza et al., 2011; Wu et al., 2009; Yamamoto et al., 2013).

In addition, after treatment, GFAP expression was not significantly changed compared to control groups. All these might be due to the relatively early age of these transgenic mice and the short term of our treatment.

Our results also showed a significant reduction in Aβ deposition in the mice treated by Hesperidin and Icariin, even following a relatively short-term treatment of 10 days. Nevertheless, the exact mechanism of reduced Aβ deposition observed in our study remains unclear; it may be attributed to the attenuated neuro-inflammation, since attenuated neuro-inflammation has been shown to contribute to reduced characteristic AD pathology, including Aβ-plaque accumulation (Tweedie et al., 2012).

Interestingly, in many other *in vivo* and *in vitro* studies, both DHM and Baicalin were reported to exert anti-inflammatory effects. However, in the current study, they could improve neither the behavioral dysfunction nor pathological changes. The reasons were manifold. Firstly, though Baicalin had the ability to traverse the BBB, the permeability was not high. For instance, after be injected at the dose of 24 mg/kg, the concentration of Baicalin, in cerebrospinal fluid (CBF) was merely 27% of that in serum (Huang et al., 2008). In contrast, Youdim et al.'s study reported that the apparent permeability to cross the *in vitro* BBB model was higher for the citrus flavonoids, especially hesperitin and naringenin, compared with their more polar glucuronidated conjugates, the dietary anthocyanins and to specific phenolic acids derived from colonic biotransformation of flavonoids, and those of epicatechin and its *in vivo* metabolites (Youdim et al., 2003).

In addition, after oral administration of Icariin at the dose of 100 mg/kg, the absolute availability was 12.02% in rats (Ye et al., 1999); while this parameter was much lower

(4.84%) in rats after giving 100 mg/kg of Baicalin by gavage (Xing et al., 2004). Moreover, the excreted amount of Icariin from urine, faeces and bile was very small, and the accumulated amount for 24 hours was merely 1.99%, 12.83% and 0.066% of the oral administration dosage (100 mg/kg) (Ye et al., 1999); in contrast, the excreted amount of DHM was a little higher as about 29.0% of the dose (100 mg/kg) was eliminated via faeces (Li, 2003).

Most importantly, evidence from a few papers showed that, to varying degrees, polyphenols might exert effects on cell signaling (Joseph et al., 2003; Kong et al., 2000; Moon et al., 2003; Schroeter et al., 2002; Stahl et al., 2002). A number of protein kinases related to signal transduction, such as mitogen-activated protein kinase (MAPK) and protein kinase C (PKC), were supposed to be most important factors of cellular regulation affected by polyphenols. If these kinases were inhibited, DNA-binding capacity of transcription factors including activator protein-1 (AP-1) and NF-κB would be modulated, and the expression rate of the gene target would be regulated (Kim et al., 2004). MAPKs comprised a group of serine/threonine kinases which were activated by multiple protein kinases in response to extracellular stimuli (Zhu et al., 2002). Three main groups of distinctly regulated MAPK cascades were known in human that would lead to the change of gene expression: ERK 1 and 2, JNK, and p38. MAPK positively regulated the expression of a number of genes involved in inflammation, including IL-1β, IL-6, IL-8, TNF-α, cyclooxygenase-2 (COX-2) and collagenase-1, -3 (Baldassare et al., 1999; Hommes et al., 2003; Karahashi et al., 2000; Kyriakis and Avruch, 2001; Manthey et al., 1998). After cerebral brain ischemia, p38α MAPK was sustained activated in activated microglia in the brain (Sugino et al., 2000). In the brains of transgenic AD mice, up-regulation of MAPK was reported, and Aβ exposure was turn out to stimulate the phosphorylation of MAPK in microglia *in vitro* (McDonald et al., 1998). Another control point of pro-inflammatory gene expression was the nuclear factor NF-κB transcriptional system, which played a fundamental role in inflammation (Pereira and Oakley, 2008). A number of kinases were activated in succession following

stimulation, and released NF-κB, which might translocate to nucleus and activate the transcription of multiple genes, such as IL-6, IL-8, TNF-α, iNOS, COX-2 and other cytokines (Barnes and Karin, 1997; Lawrence, 2009). Chronic activation of NF-κB by microglia throughout the progression of AD could be an important causative factor of inflammatory process that lead to tissue injury (Kim et al., 2006b). Through the down-regulation of NF-κB activation followed by the suppression of inhibitor-κB (I-κB) degradation and phosphorylation of JNK1/2 and p38 MAPKs after challenge with LPS, Hesperidin was shown to exert the *ex vivo* inhibitory effects on LPS-induced NO and prostaglandin E2 production, and expression of Cox-2 and iNOS in RAW 264.7 cells (Yang et al., 2012a). Another in vitro assay also showed that Hesperidin modulated neuronal cell death by activating MAPK and PI3K pathways (Nones et al., 2011). Icarin significantly inhibited the release of NO, ROS, PGE-2 and mRNA expression of pro-inflammatory cytokines including IL-1β, IL-6, TNF-α, iNOS and COX-2 in LPS-activated microglia through blocking TAK1/IKK/NF-κB and JNk/p38 MAPK pathways (Zeng et al., 2010a). Besides, as a phoshphodiesterase-5 (PDE5) inhibitor, Icariin improved learning and memory functions in a transgenic mouse model of AD possibly through the simulation of NO/cyclic guanosine monophosphate (cGMP) signaling and coordinated induction of NOS isoforms (Jin et al., 2014). Baicalin and DHM appeared to be $GABA_A$ receptor agonist. Dai et al's research suggested that Baicalin had a neuroprotective effect against delayed neuronal cell death after ischemia/reperfusion through the activation of GABAergic signaling, heat shock protein (HSP70) and MAPKs cascades. Though the remarkable induction of phosphorylated Akt and NF-κB after LPS stimulation was blocked by DHM in RAW264.7 cells, the increased phosphorylations of p38, ERK and JNK were not attenuated, suggesting the anti-inflammatory effect of DHM was based on the inhibition of ROS/Akt/IKK/NF-κB signaling pathway rather than MAPK pathway (Qi et al., 2012). Blockage of calpain activation has been shown to down-regulate BACE1 and up-regulate ATP-binding cassette transporter A1 (ABCA1), which contributed to

reduced production and increased clearance of Aβ (Medeiros et al., 2012). Besides, inhibition of calpain reduced the microglial activation and astrogliosis. As shown in Figure 6-9, calpain might be targeted by Baicalin and DHM, rather than by Hesperidin and Icariin.

Despite the healthy promotion properties of polyphenols have attracted a lot of attention, its administration *in vivo* remains problematic due to their poor water solubility and stabilities, leading to low absorptivity and bioavailability. Nanoemulsions are a group of extremely small emulsion drop-lets usually in the range of 20-200nm, which are much smaller than the sizes of normal emulsions (ranged from 1 to 100 μm) (Wang et al., 2007a). Nanoemulsions offer advantages in providing better bioavailability, prolonging retention time in blood, improving entrapment efficiency and controlling drug release (Dai et al., 2010; Jia et al., 2012; Zhao et al., 2013). Also, nanostructured carriers serve as an effective and safe delivery vehicle across oral and CNS barriers (Ganta et al., 2010).

The neuroprotection of Hesperidin and Icariin on the behavioral dysfunction and pathological changes in transgenic APP/PS1 mice have been demonstrated in this study. Moreover, many other surprising diversity of compounds and approaches with therapeutic potential in preclinical AD disease models is striking and promising (Li et al., 2013a). However, we have still a long way ahead to achieve ultimately promising therapeutics in AD capable of inhibiting progression of disease in humans. One major problem is the lack of animal models for preclinical trials that fully represent human AD pathology. Each animal model is only able to significantly present one or two major aspect of AD pathology. The point is to take advantage of specific potentials of each model for therapeutic investigations. The greatest advantage of APP/PS1 transgenic mouse model is exhibiting a rapid neuritic-type amyloid deposition at a very early age, while at the same time Aβ deposition can be detected in the cingulate and motor cortex

and hippocampus. Thus, this model is suitable to be employed in proving the potential anti-amyloidosis and anti-inflammation therapeutic effects of agents when transgenic mouse was at an early age. In terms of transgenic mice expressing tau or tau/APP, they are appropriate models for trials on cytoskeletal- and tau-oriented therapeutics (Jaturapatporn et al., 2012; Michaelis et al., 2006). The cross-bread of MCAT mice with Tg2576 presents significant mitochondrial dysfunction, making this model appropriate for general- and MCAT-oriented antioxidant studies (Mao et al., 2012a). Another challenge in preclinical studies of AD is the time course of disease in mice models as compared to humans. Transgenic mouse models are more useful for studying some aspects of initiation of AD rather than the disease process itself (Ashe and Zahs, 2010; Shi et al., 2012b). The effect of ageing and comorbidities, like diabetes or atherosclerosis, needs to be addressed in more detail. Some additional elements such as diet, social as well as environmental factors play vital roles in triggering the disease and its progression in humans. This is difficult to mimic in animals. However, the transgenic models will continue to play vital roles in preclinical and clinical trials and can be regarded as tools for developing insight into the mechanism of this debilitating disease for many more years.

Taken together, our results showed protective roles of Hesperidin and Icariin in the transgenic APP/PS1 mouse model at a relatively early stage. Hesperidin and Icariin treatment restored impairment in nesting ability of these transgenic mice. The social interactive behavior was improved after the treatment of Hesperidin. Immunohistochemical results indicated that Hesperidin and Icariin significantly ameliorated accumulation of Aβ depositions and reduced microglial activation in both cortical cortex and hippocampus. All these results reinforce the importance of neuro-inflammation in the AD pathogenesis and suggest that Hesperidin and Icariin, or potentially other polyphenolic compounds, may be considered promising therapeutic options of human AD.

Summary

Alzheimer's disease (AD) is one of the most important neurodegenerative disorders, bringing about huge medical and social burden worldwide. It is a multifactorial disease, clinically characterized by progressive cognitive loss, neuropsychiatric and behavioral disorders. Neuropathological examination of the brains of AD patients reveals extracellular amyloid beta (Aβ) plaques in brain parenchyma and increased neuro-inflammation.

Natural polyphenols, most common compounds in foods and herbal beverages, are a large class of phytochemical that are composed of aromatic and one or more phenolic rings. Four polyphenolic compounds (namely Hesperidin, Icariin, Dihydromyricetin and Baicalin) with potential neuroprotective properties were selected for further evaluation.

In the current study, potential therapeutic effects of these four polyphenols were evaluated in the transgenic APP/PS1 mouse model of cerebral amyloidosis. 5-months old transgenic mice were treated by Hesperidin, Icariin, Dihydromyricetin, Baicalin (100 mg/kg body weight) or vehicle by gavage, respectively. Therapeutic effects of these polyphenols were monitored by behavioral tests of nesting construction and social interaction. Then, the mice were sacrificed and tissues were taken for the pathological investigation.

After a relatively short-term treatment of 10 days, Hesperidin and Icariin treatment significantly restored deficits in nesting ability, in comparison to age-, gender-, and bodyweight- matched transgenic littermates. The social interactive behavior was improved after treatment with Hesperidin. Immunohistological results indicated that Hesperidin and Icariin significantly attenuated β-amyloid deposition and microglial activation in both cortex and hippocampus of these transgenic mice. However, neither the behavioral dysfunction nor histopathological changes were improved after the treatment of Dihydromyricetin and Baicalin.

Our findings suggest that Hesperidin and Icariin might be considered potential therapeutic candidates of human AD or even other neurodegenerative diseases.

Zusammenfassung

Morbus Alzheimer (AD) ist eine schwere neurodegenerative Erkrankung, die weltweit große medizinische und soziale Probleme verursacht. Diese multifaktorielle Erkrankung, die sich klinisch besonders durch den Verlust kognitiver Fähigkeiten auszeichnet, zeigt auch viele neuropsychiatrische Störungen und Verhaltensauffälligkeiten. Neuropathologisch charakteristisch sind die extrazellulären Amyloid-β (Aβ) Plaques im Gehirnparenchym und eine lokale Entzündungsreaktion.

Natürliche Polyphenole, häufige Komponenten von Nahrungsmitteln und pflanzlichen Getränken, bilden eine große Gruppe von Phytochemikalien, die strukturell aus aromatischen/penolischen Ringsystemen und Gruppen bestehen. Vier Polyphenole (Hesperidin, Icariin, Dihydromyricetin und Baicalin) mit potenziellen neuroprotektiven Wirkungen wurden für unsere Untersuchungen ausgewählt.

Die therapeutischen Effekte dieser vier Polyphenole wurden im transgenen APP/PS1 Mausmodell der zerebralen Amyloidose untersucht. Fünf Monate alte transgene Mäuse wurden mit Hesperidin, Icariin, Dihydromyricetin, Baicalin (100 mg/kg Körpergewicht) behandelt. Die therapeutischen Wirkungen wurden zuerst durch Verhaltensanalysen, wie Nestbau und soziale Interaktion, untersucht. Danach wurden die Mäuse getötet und Gewebe für die pathologische Untersuchung aufbereitet.

Nach einer relativ kurzen Behandlungszeit von 10 Tagen verbesserten Hesperidin und Icariin signifikant den Nestbau der transgenen Mäuse. Zusätzlich wurde die soziale Interaktion durch Hesperidin verbessert. Die Ergebnisse der Immunhistologie zeigten, dass Hesperidin und Icariin die Aß-Ablagerungen im Cortex und Hippocampus signifikant reduzierten. Baicalin und Dihydromyricetin verbesserten beide nicht die Verhaltensstörungen oder die histopathologischen Veränderungen.

Unsere Untersuchungen legen nahe, dass insbesondere Hesperidin und Icariin potenzielle Kandidaten für die Behandlung von AD und vielleicht sogar anderen neurodegenerativen Erkrankungen sind.

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List of publications

Parts of contents in the thesis are derived from articles published or submitted for publication.

Chaoyun Li, and Hermann J. Schluesener. Health-promoting effects of the citrus flavanone hesperidin. Crit Rev Food Sci Nutr (accepted).

Chaoyun Li, Kunxiong Yuan, and Hermann J. Schluesener. Impact of minocycline on neurodegenerative diseases in rodents: a meta-analysis. Revneuro, 2013; 24(5): 553-562.

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Chaoyun Li, Caroline Zug, Hongchun Qu, Hermann J. Schluesener, and Zhiyuan Zhang. Protective effects of Hesperidin upon behavioral impairment and neuropathology in the transgenic APP/PS1 mouse model. (Submitted)

Zhiyuan Zhang, Caroline Zug, **Chaoyun Li**, and Hermann J. Schluesener. Icariin ameliorates neuropathological changes, TGF-ß1 accumulation and behavioral deficits in a mouse model of cerebral amyloidosis. (Submitted)

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Curriculum vitae

Basic information

Name: Li Chaoyun

Sex: Male

Date of Birth: December 12, 1987

Place of Birth: Henan, P.R. China

Education experience:

1993.09-1998.07	Primary School, Taiqian, Henan Province, P.R. China
1998.09-2001.07	Junior High School, Taiqian, Henan Province, P.R. China
2001.09-2004.07	Senior High School, Puyang, Henan Province, P.R. China
2004.09-2008.07	Department of Labor and Social Security, School of Public Health, Southeast University, Nanjing, Jiangsu Province, P.R. China
2008.09-2011.07	Department of Epidemiology and Biostatistics, School of Public Health, Southeast University, Nanjing, Jiangsu Province, P.R. China
2011.09-present	Ph. D. candidate of Prof. Dr. Hermann J. Schluesener, Institute of Pathology and Neuropathology, University of Tuebingen, Tuebingen, Germany

Preliminary research experience:

2006.01-2006.12	Student Research Training Program of Southeast University "Research on the development of insurance market at county level."
2007.01-2007.12	Student Research Training Program of Southeast University "Evaluation on the Ability of Jiangsu Regional Sustainable

	Development Based on Mapinfo"
2008.01-2010.12	National Student Research and Innovation Program of China "Construction of Dietary Exposure Evaluation Model"
2008.09-2009.05	National Science and Technology Support Program of China "Research on exposure assessment of chemical pollutants"
2009.08-2009.10	Clinical trial of H1N1 vaccine, Jiangsu CDC, P.R. China
2009.10-2009.11	Clinical trial of Oral polio vaccine, Jiangsu CDC, P.R. China
2010.07-2010.08	Clinical trial of Cholera vaccine (Intestines dissolving capsule), Academy of Military Medical Science, P.R. China
2010.08-2010.09	Clinical trial of Anthrax vaccine, Academy of Military Medical Science, P.R. China
2011.09-present	Preclinical trials using herbal extract on transgenic Alzheimer's disease mice and rats with experimental autoimmune neuritis
2013.09-2013.11	Visiting student in the Life Science and Technology School, Tongji University, Shanghai, P.R. China, supported by DAAD