Ampelopsin alleviates cognitive impairment of SAMP8 mice by inhibiting microglial polarization and NLRP3 degradation via autophagy

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Research Article

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Abstract

Microglial polarization and NLRP3 inflammasome mediated inflammation response are known to be involved in the pathological procession of AD. Ampelopsin, a natural flavonoid compound from Chinese herb *Ampelopsis grossedentata*, has been reported to have neuroprotective functions. However, there have been no reports on whether DHM suppresses microglial polarization and NLRP3-Caspase-1 inflammasome via autophagy pathway in an Alzheimer’s disease model. We aimed to study the effects of ampelopsin on M1/M2 polarization and the mechanism to regulate anti-inflammation both in vivo and vitro models. BV2 cells were treated with LPS in the presence or absence of DHM, and SAMP8 mice were orally administered 100 or 200 mg/kg/day of DHM for 8 weeks. Our results showed that ampelopsin significantly mitigated cognitive impairment and AD-like pathological proteins BACE1 and APP levels in AD mice. Treatment with different dose of ampelopsin efficiently suppressed NLRP3-Caspase-1 inflammasome activation, IL-1β and IL-18 production as well as microglia activation in the hippocampus of SAMP8 mice. Mechanistically, DHM promoted the transition from M1 to M2 microglia by up-regulating SIRT1 signaling. Transmission electron microscopy results further confirmed that DHM reversed impaired autophagy in AD mice. However, CQ, as an autophagy inhibitor, not only blocked the above protective effects of DHM in vivo, but also exacerbated those pathological changes. Our findings reveals activation of autophagic induced by DHM promote M2 polarization, NLRP3 inflammasome degradation, inhibiting inflammatory response, in turn, improving cognitive function in SAMP8 mice.

1. Introduction

Aging is a major risk factor for the development of several neurodegenerative diseases including Alzheimer’s disease (AD). Inflammation and defective autophagy are associated with AD [1, 2]. As one of the major type of late-life dementia, AD shows the characteristics of pathological features, such as the presence of β-amyloid plaques and neurofibrillary tangles as well as neuroinflammation accompanied by progressive cognitive decline [3]. AD inflicts over 50 million people in the world and it is the 5th main cause of death [4]. However, the molecular mechanisms of AD are still unknown.

Some evidence indicates that neuroinflammation mediated by microglia was an important feature of AD[5, 6]. As the innate immune effector cells of the central nervous system, microglia, are involved in two aspects of AD removal of extracellular Aβ by phagocytosis and production and release of cytokines resulting in progressive neuroinflammation, and these roles are be closely related to different functional phenotypes of microglia: M1(neurotoxic, classic pro-inflammatory) and M2 (neuroprotective, anti-inflammatory) phenotype. Thus, based on this theory, NSAID (nonsteroidal anti-inflammatory drugs) treatment may potentially slow the development of AD. However, results from trials of symptomatic subjects were disappointing, and the only NSAIDs prevention trial was suspended because of adverse side effects [7]. The NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome in NLR related inflammasomes is one of the most widely investigated. The activation of NLRP3 inflammasome could recruit and activate Caspase-1, resulting in the secretion of pro-inflammatory cytokines, such as mature IL-1β and IL-18[8]. Overactivation of NLRP3 inflammasome is involved in the pathogenesis of
several neurodegenerative diseases[9, 10]. Currently, the unequivocal upstream activation mechanism of NLRP3 inflammasome remains unclear. To date, no preventive or neuroprotective therapies for AD exist. Therefore, targeting the activity of the NLRP3 inflammasome would be a potential and novel therapy for AD.

Autophagy, as a lysosome-dependent degradation pathway, plays indispensable roles in the maintenance of quality control of essential cellular components by degrading long-lived proteins, cytosolic components, or damaged organelles. Under stress and starvation conditions, autophagy can be activated. Autophagy is regulated by several autophagy-related genes/proteins. For example, Beclin1, is an initiator of autophagy and required for autophagosome formation. During the formation of autophagosomes, LC3-I is processed to LC3-II by lipidation, therefore, the ratio of LC3II/I can be used as a classic marker protein. p62 mediates the degradation of target proteins and the binding of damaged organelles to the autophagosome, thus serving as an index of autophagy flux. Increasing evidence demonstrated that impaired or dysfunctional autophagy leads to excessive inflammation[11]. In recent years, accumulating evidence demonstrates that autophagy negatively regulates the activation of the NLRP3 inflammasome, thereby inhibiting the inflammatory response of the body and reducing inflammatory injury of tissues in response to diseases. Subsequent studies have implicated that deficient autophagy or impaired autophagic flux can result in neurological damage in aging and age-associated neurodegenerative diseases, conversely, autophagy inducers have been reported to play a neuroprotective role[12-14]. In line with this, our previous studies have shown that autophagy activation could ameliorate the dysfunction of cognitive in different AD animal models and natural aging rats[15-17]. Although some evidences demonstrated that autophagy regulates NLRP3 inflammasome and alleviating inflammation[18-21]. Considering that AD and PD are both neurodegenerative diseases, whereas the underlying mechanism of how autophagy affects the activation of NLRP3 inflammasome in AD is not completely understood. Above findings encourages us to speculate that inhibiting NLRP3 inflammasome via pharmacological activation of autophagy may be a potential therapeutic strategy in AD.

Interestingly, natural extracts or derivatives from herbs have attracted great attention due to their wide range of biological activities and low toxicity. Ampelopsin, alternate name dihydromyricetin (DHM), is a natural flavonoid from Rattan tea and has long history as a medicinal and edible plant in China[22]. Its chemical structure is shown in [23]. DHM has been reported to possess anti-inflammatory, anti-oxidative, and anti-tumor activities[24]. Our team demonstrated that DHM execute its protective function against 6-OHDA-induced neurotoxicity in PD cell models[22]. Furthermore, our recent study revealed that DHM can attenuate brain aging induced by D-gal, ameliorate pathological characteristics of AD, and improve cognitive capacity[15]. These findings suggest that DHM is a promising candidate for the prevention and treatment of neurodegenerative diseases. Although a study[25] have reported that DHM alleviates inflammatory cytokines in serum and hippocampus of AD models with Aβtreated. There have been no reports on the role of DHM on M2 polarization is mediated via the activation of Sirt1/autophagy. Additionally, the effect of DHM on autophagy and its relationship with the NLRP3 inflammasome has not
yet been investigated in senescence-accelerated prone-8 (SAMP8) mice, a well-established model with age-related phenotypes.

In this study, we investigated the effects of DHM in regulating the NLRP3 inflammasome, M1/M2 shift of microglia and autophagy both in LPS-treated microglial cells and SAMP8 mice sought to determine the role of Sirt1/autophagy in the DHM mediated effects.

## 2 Material And Methods

### 2.1 Reagents

Ampelopsin (purity of more than 98%) was obtained from Aladdin Industrial Corporation. Chloroquine was obtained from Sigma. Primary antibodies including NLRP3, cleaved Caspase 1, Iba-1, CD206, IL-4, Arg1, iNOS, TNF-α, IL-6, LC3, p62, Beclin 1, Sirt1, GAPDH were purchased from Cell Signaling Technology (Danvers, MA, USA). Secondary antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA).

### 2.2 Animals and treatments

Totally 20 male SAMP8 mice (age: 6 months old; body weight: 30 ± 5 g; c) were obtained from Beijing Huafukang Company (Beijing, China). All mice were kept in standard environment with room temperature of 22 ± 2 °C and a dark-light cycle (12 h: 12h), and accessed to food and water freely. The protocols were reviewed and approved by Institutional Animal Care and Use Committee at Wuhan Sports University. The mice were randomly divided into four groups including SAMP8 group, low dose DHM(100mg/kg/ group, D1), high dose DHM(200mg/kg/ group, D2) and high dose DHM plus Chloroquine(DHM+CQ) with 10 mice in each group. Age-matched (senescence accelerated mouse resistant 1, SAMR1) as a negative control. The mice from DHM groups were administered with DHM dissolved in DMSO at the designated dosages by gavage once a day at 9:00am for 8 consecutive weeks. The mice from the SAMP8 group were received same volume of DMSO. Each administration of Chloroquine should be 2 h ahead of DHM.

### 2.3 Behavioral testing

After 8 weeks intervention, morris water maze (MWM) test was used to evaluate spatial learning and memory capacity of SAMP8 mice. The MWM test lasts for a total of 5 days. The first day represents the positioning test. Mice were released into water at different starting points and subjected to four trials per day to find the platform. Mice that failed to find the platform within 60 s were directed to the platform and allowed to stay for 15 s. On the last day, mice underwent the space-exploring test in which the platform was removed. Mice were allowed to swim freely for 60 s. The escape latency that mice spent in the target platform and the swimming speed were recorded by computer.

### 2.4 Preparation of brain tissues and histological examination of hippocampus
After MWM task, 5 mice were decapitated under anesthesia. The hippocampal tissues were isolated and frozen in liquid nitrogen or -80°C. In addition, some frozen brains were embedded, cut into sections with 20mm thick along the coronal plane, then placed on slides. At last, slides were fixed with acetone for 10 min at 4°C, allowed to dry at room temperature, and then stored at -20°C until further staining.

### 2.5 Transmission electron microscopy assay

After MWM tests, mice were anesthetized with 10% chloral hydrate and were decapitated. The right hippocampal tissues were harvested and fixed in phosphate buffer for 2 h, rinsed with 1 mmol/L phosphoric acid solution, subsequently then fixed in 1 % osmium tetroxide for 2-3 h. The block was cut into sections with 70 nm thick. The sections were stained with 3 % uranyl acetate and lead citrate and then examined under HT-7700 transmission electron microscope (HITACHI Hi-technology, Japan) in Wuhan University. Autophagosomes were calculated as follows: 50 different cell sections were observed under TEM and the total number of autophagosomes and lipofuscin in hippocampal neurons was calculated.

### 2.6 Cell culture

BV2 mouse microglia cell line purchased from the American Type Culture Collection (ATCC, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, USA) supplemented with 10 % fetal bovine serum (FBS; Gibco, USA) in a 5% CO2 incubator at 37°C. 60%-80% confluent cells were divided into five groups and cultured as the following different treatments: Control, LPS+ATP, LPS+ATP+D1, LPS+ATP+D2, LPS+ATP+D3. Next, the BV2 cell line were pretreated with different dose DHM(6.25, 12.5 and 50μg/mL) or Ex527 for 2 h and then incubated with 1μg /mL LPS for 24 h, prior to 1mM ATP stimulate 30 minutes.

### 2.7 MDC staining

The protocols for MDC staining were reported according to the manufacturer's instructions. BV2 cell were treated by DHM or LPS, then incubated with MDC dye solution with concentration of 50uM At 37 °C for 15 min. After three times of PBS cleaning, the cells were fixed with 4% paraformaldehyde at room temperature for 30 minutes. Finally, all stained cover glasses were rinsed with PBS for three times, and then sealed with neutral gum, and imaged with 380 nm emission wavelength under fluorescence microscope. For quantitative analysis of fluorescence intensity, measure the IOD value with Image ProPlus 6.0 software.

### 2.8 Western blotting analysis

The hippocampus tissue were acquired after the end of MWM tests. The tissues were homogenized in lysis buffer for 30 min on ice and centrifuged at 12000 rpm for 30 min. The protein quantification was carried out using a BCA kit (Walterson Biotechnology Inc., Beijing, China). The protein samples were boiled in the presence of sample buffer at 95 °C for 5 min before western blotting. The target protein was separated by SDS-PAGE gel, transferred to PVDF membrane, blocking the membranes with 5% milk
solution for 1 h, the blot then probed by corresponding primary and secondary antibodies. Finally, the target protein was visualized by enhanced chemiluminescence (ECL) reagent and imaged by ultrasensitive fluorescence/chemiluminescence imaging system ChemiScope6300 (CLiNX Science Instruments, Shanghai, China)

2.9 Statistical analysis

The results are expressed as mean ± standard deviation (M ± SD) at least three independent experiments. Data were analyzed statistically using GraphPad Prism software. The comparisons between the groups were determined using Two-way analysis of variance (ANOVA) followed by Tukey’s post hoc tests when necessary. P Values of < 0.05 were considered significant.

3. Results

3.1 NLRP3 inflammasome activation and autophagy impairment are associated with AD process

SAMP8, as a model for studying AD, which shows pathological features such as age-related cognitive deficits and abnormal pathological proteins observed in patients with AD[26, 27]. SAMR1 is a negative control. It is accepted that aging is usually accompanied by up-regulated neuroinflammation and low-grade inflammation has been reported to contribute the development of degenerative changes in the brain, in turn triggering neurodegenerative disorders pathology[28]. To determine the effect of age on NLRP3 inflammasome activation, we measured the expression of core multiprotein complex of the canonical inflammasome (NLRP3 and Cleaved -Caspase1) in SAMP8 mice by Western blotting. Compared with SAMR1 mice, increased NLRP3 and cleaved-Caspase1 protein levels were observed in the SAMP8 mice (Figure 1), which reveals that the NLRP3 inflammasome is activated during the pathological process of AD. Besides, mice in SAMP8 group exhibited a significant autophagy-related protein reduction in the expression of Beclin1, LC3I and p62. These data suggest that NLRP3 inflammasome activation is negatively related with impaired autophagy in SAMP8 mice, which prompts us to test whether activating autophagy has a protective role in SAMP8 mice.

3.2 DHM inhibited LPS induced NLRP3 inflammasome in vitro

Considering the relationship between NLRP3 inflammasome activation and impairment of autophagy, we next determine whether DHM alone activates autophagy in classical microglia cells BV2. As shown in Figure 2 A and B, Microglial autophagy was up-regulated by DHM alone with concentration 6.25,12.5 and 25μg/μL. In addition, immunofluorescence imaging results showed that DHM treatment enhance the number of GFP-LC3 puncta per cells. Furthermore, the BV2 cells were subjected for 24 h to acute proinflammatory cytokine LPS (1μg/ml) followed by 30 min of ATP. As shown in Figure 3A and B, western blotting indicated that LPS induced increased NLRP3, Cleaved-Caspase1, IL-1β and IL-18. In contrast, different dose of DHM almost reversed the inflammation to the control level, immunofluorescence imaging results also verified that DHM reversed LPS-induced impaired autophagy.
3.3 DHM ameliorates the activation of NLRP3 inflammasome and microglia activation in hippocampus of SAMP8 mice

Having characterized the effects of DHM in vitro, we then examine the activity of DHM in an SAMP8 mouse model. As shown in Figure 4, similar with vitro, western blotting data showed that increased NLRP3, cleaved-Caspase1, mature IL1β and IL-18 in SAMP8 mice, conversely, different dose of DHM was able to effectively reduce above increased protein levels in a dose-dependent manner. To verify the relationship between microglia and the NLRP3 inflammasome, microglia activation was evaluated by western blotting with the classic antibody specific for Iba-1. We found Iba-1 protein level was significantly suppressed by DHM treatment compared with SAMP8 group. These data indicated the infiltration of microglia in hippocampus of mice.

3.4 A lysosome inhibitor, CQ blocked the anti-inflammarogy effects of DHM in AD mice

Increasing studies have confirmed that autophagy defects cause neurodegeneration in mice[29-31], and the activation of autophagy can help delay aging process. Hence, to investigate the autophagic activity in the hippocampus of SAMP8 mice, we monitored the dynamic changes of ATG7, p62, Beclin1 and the ratio of LC3-II/LC3-I by Western blotting. As shown in Figure 5A, compared with SAMP8 group, increased ATG7 and Beclin1, decreased p62 protein levels were observed both D1 and D2 groups, and the ratio of LC3-II/LC3-I had an increasing trend, indicating that different doses of DHM treatment could improve the formation of autophagosomes and impaired autophagy flux of SAMP8 mice. In line with western blotting, the representative images of transmission electron microscopy (TEM) showed more formation of autophagosome (yellow triangle) in hippocampus of mice from D1, and D2 groups (Figure 5B). In addition, a deacetylase SIRT1 protein that influences microglial activation was upregulated by DHM treatment.

Since the autophagy-lysosome pathway is involved in protein degradation, thus, we speculated whether DHM activated autophagy accounts for NLRP3 degradation. To further assess whether upregulation of autophagy affects NLRP3 inflammasome activation and IL-1β as well as IL-18 production in SAMP8 mice. Thus, mice were pre-treated with CQ that blocks the association of autophagosomes and lysosomes. As shown in Fig.5A and B, DHM combined with CQ treatment markedly decreased the protein level of ATG7, Beclin1 and LC3 II/I, as well as the number of the autophagosome compared to DHM group. Besides, the level of p62 was increased by CQ treatment. As we expected, treatment both CQ and DHM effectively blocked DHM induced NLRP3 degradation and caspase-1 inactivation compared with DHM treated alone mice. Moreover, IL-18 and IL-1β production also were significantly enhanced in D2Q group, supporting NLRP3 degradation via autophagy (Figure 5C).

3.5 DHM promoted the transition from M1 to M2 Microglia

To further identify the role of microglial polarization in the protective effects of DHM in SAMP8 model, the phenotype of microglia was detected in mice with AD with or without DHM treatment. As shown in Figure 5, SAMP8 mice displayed higher inducible nitric oxide synthase (iNOS, M1 phenotype biomarker)
and lower CD206 and Interleukin 4 (IL-4), the markers of M2 microglia. Interestingly, DHM treatment significantly reduced iNOS and increases M2 biomarkers when compared to SAMP8 group, suggesting that DHM treatment was able to promote M2 phenotypic transformation in the hippocampus of AD mice. Having determined the effects of DHM on microglial polarization, we next evaluated the effects of DHM for cytokines release. Similar results of proinflammatory cytokines were found in the hippocampus. Results showed that DHM significantly decreased the protein levels of IL-6 and TNFα in the hippocampus of mice. However, these pro-inflammatory cytokines were significantly promoted by CQ. Consistent with exacerbating neuroinflammatory effects of CQ treatment in SAMP8 mice, mice of D2Q group also displayed low CD206 and IL-4 levels. The data indicated that DHM-activated autophagy facilitated M2 polarization and inhibited M1 polarization of microglia in SAMP8 mice.

3.6 SIRT1 inhibitor, EX527 blocked DHM effects in BV2 cell lines

To further identify whether DHM promote microglial polarization is dependent on Sirt1, the Sirt1 selective inhibitor, EX527 was used in following vitro experiment. Above experiments were subsequently repeated in the presence of EX527. The data demonstrated that the anti-inflammatory action of DHM was blocked by EX527, as illustrated by the upregulation of NLRP3 and cleaved Caspase-1 after EX527 addition (Figure 7), indicating that DHM reduced NLRP3 was in part mediated by SIRT1. These data suggest that the regulatory effect of DHM in microglia inflammation is positively associated with Sirt1 signaling in SAMP8 mice.

3.7 DHM treatment markedly reduced AD-like pathology and rescued learning and memory decline of SAMP8 mice

To ascertain whether neuroinflammasome activation is responsible for the neuroprotective effects of DHM. Neuronal morphology was determined using Nissl staining. Representative images of Nissl staining were shown in Figure 8E, the neuronal cells in the SAMP8 group were loosely arranged, most of the nuclei were deeply stained. Conversely, both two different dose DHM treatment significantly increased the number of Nissl bodies and alleviated neuron damage. Interestingly, the arrangement of neurons in D2Q group was not as neat as that of mice in the D2 group, with hyperchromatic nuclei, and the number of Nissl bodies was also significantly reduced compared with D2 alone group.

β-site amyloid precursor protein cleaving enzyme 1 (BACE1) is a key enzyme responsible for Aβ formation, which dysregulation resulting in excess Aβ deposition, in turn implicated in sporadic AD. In addition, abnormal cleavage of amyloid precursor protein (APP) is also closely related to AD. In order to explore whether DHM treatment affects above proteins levels, western blotting was performed with BACE1 and APP by the corresponding antibody. The data reveals data both D1 and D2 group mice had decreased levels of APP and BACE1. Besides, although there was no significant difference between DHM group and SAMP8 group, DHM did show a trend of toward decreasing the expression of p-tau. In contrast, CQ plus DHM treatment reversed BACE1 and APP decline compared with D2 group.
To further validate the neuroprotective role of DHM in AD mice, MWM test was used to evaluate the spatial learning and memory function of different group mice. Compared to SAMP8 group, the escape latency was obviously shortened at the fourth and fifth day both in D1 and D2 group (Figure 8A and B), indicating the learning ability is improved. At the fifth day, the number of crossing target platform increased both in D1 and D2 group compared to the SAMP8 group (Figure 8C). More importantly, the neuroprotective effects of DHM as evidenced by improvement of learning and memory performance was blocked by supplement with CQ plus DHM (Fig. 8A and D). These data indicates that DHM exerts neuroprotective effects in AD mice by up-regulating autophagy.

4 Discussion

AD, as a common, age-related neurodegenerative disease is a major cause of dementia. NLRP3 have been recognized as a promising biomarker for AD, the precise mechanism of NLRP3 in AD is not yet been fully elucidated. In the present study, we identified Sirt1-autophagy signaling as a regulator of the microglial NLRP3 inflammasome pathway, leading to cognitive dysfunction in an AD-like mouse model. To unveil the impact of Sirt1 on microglial activation, we analyzed mouse BV2 cell lines, coupled with SAMP8 mice, underlines the role of SIRT1 regulation in the pro-inflammatory and inflammasome pathway. We found DHM can inhibit NLRP3 inflammasome activation, microglia polarization via autophagic signaling, in turn improving cognitive impairment of AD mice.

Neuroinflammation is the third pathological hallmarks after Aβ plaques and intracellular neurofibrillary tangles in the pathogenesis of AD. Whether inflammation is an initiating factor or consequence of AD is still unclear. In the current study, we observed that activation of NLRP3 inflammasome in microglia led to neuron degeneration and learning and memory decline in mice. Furthermore, in line with previous studies[32], we observed that increased NLRP3 and cleaved Caspase1, in turn, releasing mature IL1β and IL-18 in SAMP8 group compared with SAMR1 group. Impaired autophagy were exhibited in SAMP8 mice, indicating that NLRP3 may a target of autophagy. Autophagy can eliminate misfolded or aggregated proteins, to maintain cellular homeostasis under physiological conditions. However, autophagy deregulation possibly cause protein aggregates, which are the main cause of neurodegenerative diseases. Recent studies have demonstrated that autophagic activity is a very close association of the activation of NLRP3 inflammasome[18, 19]. On the one hand, enhancing autophagic activity could decrease NLRP3 inflammasome activation[33]. However, impaired autophagy may lead to enhanced NLRP3 inflammasome level. Our team shown that DHM could activate autophagic flux process and alleviates dysfunction of cognitive in D-gal induced AD rats[15]. Moreover, our recent study also revealed that activation of autophagic by treadmill running could also ameliorate the decline of learning and memory ability in 21 months natural aging rats[17]. However, for elder who do not like to exercise or who cannot tolerate rigorous or regular exercise due to illness, consumption of DHM-rich tea may be a good choice. Based on the research that impaired autophagy has been related to the pathogenesis of AD and our previous publication of DHM preserving cognitive function, Hence, we speculate whether DHM exerts anti-inflammatory effects by modulating autophagy in SAMP8 mice.
To explore the potential links between autophagy and NLRP3 inflammasome activity in AD mice model, the autophagy associated protein were measured in the current study. The data from western blotting revealed that autophagic process in the hippocampus tissues, such as, initiation of autophagy, autophagosome formation and autophagosome degradation was founded to decline compared with SAMR1 group. Lucin and colleagues[34] reported that microglia isolated from human AD patients show significantly reduced Beclin1 protein. Consistent with this, we observed decreased level of Beclin1 and the ratio of LC3 II/I, but accumulation of p62. However, DHM supplementation exerted a comparable effect on autophagy induction. Furthermore, TEM results showed that the neurons of SAMP8 mice were presenting cytoplasm vacuolization, membrane ridges disappeared and lipofuscin accumulations (red triangle). As we expected, different dose of DHM ameliorates the mitochondrial damage and increases the number of autophagosomes.

Judith et al. [19] reported that selective autophagy negatively regulate microglia activation by modulating IL-1β and IL-18 production via NLRP3 degradation. In this study, autophagy inhibition by CQ blocked the anti-inflammation effects of DHM. These data in vivo suggesting the mechanism of DHM on inhibiting NLRP3 inflammasome may be attributed to the induction of autophagy. To the best our knowledge, it is plausible that impaired or insufficient autophagy cause excessive NLRP3 inflammasome mediated mature IL1β and IL-18 production, which further aggravated inflammation and neuronal cell death. Autophagy pathway could be the upstream of NLRP3 inflammasome complex. Some disease models have confirmed the above assumption. Experimental data from in vitro macrophage studies show that inhibition autophagy by 3-MA caused enhanced IL-1β formation[35, 36]. Some studies describe knockdown of autophagy genes also resulted in increased activation of the BV2 cell line or primary microglia[37, 38]. It cannot be ignored that there are somewhat controversial regarding the relationship between autophagy inhibition and the NLRP3 inflammasome. For example, Francois et a have revealed there are no changes in cytokine release after blocking autophagic flux with Bafilomycin A1[39]. We speculate that this is may be related to the autophagy state of cells under different conditions.

Previous research has mostly focused on neurons in AD, but considering microglia are the principal neuroinflammatory cells, robust microglial activation may result in the overactive of the NLRP3 inflammasome in the brain, they are gradually attracting attention. Resting microglia can respond to microenvironmental disturbance by altering its phenotypes and functions. Aging plays an important role in balancing M1 and M2 phenotypes. On the one hand, aging enhanced the M1 phenotype with excessive pro-inflammatory factors, on the other hand, it reduced the activation of M2 microglia with a decline of M2 markers [40]. Furthermore, it has been reported that Aβ and tau result in a hyper-activated M1 phenotype of microglial cells, in turn leading to detrimental brain damage accompanied by the expression of pro-inflammatory cytokines in AD. Conversely, M2 activation microglia releases beneficial factors leads to neuroprotection effect[41]. Prokop et al[42] demonstrated an excessive accumulation of microglia around amyloid plaques both in animal models of AD-like pathology and postmortem brains of AD patients. Further research demonstrated that microglial activation is closely related with markers of tau pathology[43]. Another study also revealed that microglial activation is related to the clinical dementia rating score in AD patients[44]. Thus, modulating microglial activation from M1 to M2 phenotype in AD
could be a potential therapeutic strategy. Several bioactive compounds have been reported to ascertain their ability to regulate microglial polarization, and thereby exert neuroprotection against neurodegenerative diseases in different animal models. For example, Malibatol A, a natural resveratrol oligomer extracted from the leaves of the Chinese plant *Hopea hainanensis*, has been proved to decrease the expression of M1 (CD16, CD32, and CD86), while increasing that of M2 markers (CD206 and YM-1) [45]. Platycodigenin and Curcumin could shift microglia from M1 to M2 in LPS-stimulated BV2 and primary microglia[46, 47]. Similar to aforementioned, here, we demonstrated that gene expression of pro-inflammatory M1 factors such as iNOS, TNF-a, IL-1β, and IL-6 was downregulated by DHM in the hippocampus of SAMP8 mice. Some studies have also shown that M2-polarized microglia may exert neuroprotective effects in animal and cell models of AD[48–50]. In the current study, we found that DHM treatment induced a shift in the phenotype of microglia from M1 to M2 by downregulating M1 markers (iNOS, IL-βand IL-6 ), which were found to be increased in SAMP8 group and elevated M2 markers (IL-4, Arg1 and CD206).

Next, we want to clarify the molecular mechanism of DHM promoting M2 polarization in AD. Sirtuin (Sirt1), a member of the class III histone/protein deacetylases, is involved in inflammation and cellular senescence. Previous studies identified that the overexpression of the Sirt1 in the brain reduces central nervous system AD pathologies and inhibits NF-κB signaling as well as reduces microglia-dependent Aβ toxicity[51, 52]. Thus, we determined the Sirt1 expression in AD mice, and our data showed that DHM significantly upregulated the SIRT1 expression both in vivo and in vitro. Besides, in LPS-stimulated BV2 cell, EX527 pretreatment blocked the anti-inflammatory effects of DHM, indicating that the effects of DHM on microglial polarization and neuroinflammaion are mediated at least partially by SIRT1 pathway. This study was the first report of the polarizing effect on microglia of DHM both in vivo and in vitro models of AD. These findings strongly suggested the critical role of neuroinflamasome activity and microglia polarization in AD, and inhibition of neuroinflammation might be a potential target to hinder those abnormalities.

Increasing studies shown that rapamycin improve cognitive decline in AD mice by up-regulating autophagy, eliminating Aβ deposition[53, 54]. Similar observations are gained in our recent studies. For instance, we found DHM supplementation exerts beneficial effects via the induction of autophagy in animal models of brain aging rats, illustrated that autophagy activation mediated by DHM appears to be one of the important mechanisms in brain aging and age-related diseases. Inflammation mediated by microglia was involved in the formation of Aβ and tau aggregates, as well as neuronal loss contributing to the neurodegeneration[55]. Neuronal loss in the hippocampus leads to degenerative pathological changes in AD, which, in turn, affects cognitive ability[56, 57]. So, we next investigated whether DHM supplementation could ameliorate cognitive impairment and abnormal AD-like pathological proteins. Here, we found that different dose of DHM treatment effectively reduced accumulation of APP and BACE1 proteins among SAMP8 mice, which is similar to what occurs in the brain of patients. Moreover, similar with previous study[58], we also found that application of DHM also resulted in improvement of cognitive function in SAMP8 mice. In line with our data, another study showed that DHM and Sal B ameliorates α-synuclein accumulation and aggregate formation and augmenting activation of
Chaperone-mediated autophagy[59]. Conversely, DHM plus CQ exacerbated the dysfunction of autophagy, in which promoted neuronal loss, and memory decline, as well as AD-like pathological proteins accumulation. Thus, this study provided first evidence on the positive effects of DHM on the autophagy in the hippocampus of the SAMP8 mice. Additionally, in order to further explore whether the improvement of learning and memory ability of mice by DHM is related to the improvement of motor ability, the swimming speed of mice was recorded automatically. However, no differences were observed among the four groups (data not shown), implying that the neuroprotective effect of DHM on SAMP8 mice may not be related to exercise ability. To the best of our knowledge, this is the first study to demonstrate the effects of DHM in shifting the M1/M2 polarization of microglia via activation of Sirt1/autophagy in SAMP8 mice. However, there are some limitations for this study. Firstly, we cannot exclude the possibility that the prefrontal cortex could participate in cognitive function of mice. Secondly, the crosstalk between autophagy and NLRP3 inflammasome in autophagy gene knockout mice remains to be unraveled. It will be investigated other in the future.

**Conclusion**

Taken together, the study emphasize that DHM could ameliorate AD-like pathophysiology through the promotion of microglial M2 polarization via the SIRT1/autophagy pathway in an AD model, which rescue the declined learning and memory of AD mice. Our data provides a new basis for the beneficial effects of DHM in age-associated cognitive dysfunction.

**Declarations**

**Ethics Approval and Consent to Participate** The authors declare that they have given their consent to the scientific contents and authorship to this manuscript. The protocols were reviewed and approved by Institutional Animal Care and Use Committee at Wuhan Sports University.

**Consent for Publication** Yes, all the authors have given their consent for the publication of this manuscript in its present form.

**Data Availability** All the data and results obtained during the current study are available from the corresponding author on reasonable request.

**Competing Interests** The authors declare no competing interests.

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**Author contributions** Xianjuan Kou designed experiments. Meng Zhang, Xingran Liu and Jingru Chang performed experiments, Dandan Chen and Shuaiwei Qian collected and processed data. Meng Zhang wrote the manuscript.
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References

7. !!! INVALID CITATION !!! (2009)

Figures
Figure 1

The NLRP3 inflammasomes expression both in the hippocampus of SAMR1 and SAMP8 mice. The expression level of NLRP3 and cleaved-Caspase1 proteins from two groups was measured by Western blotting (n = 3/group). △△△p < 0.001 relative to the SAMR1 group.
Figure 2

DHM alone activated autophagy in BV2 cells. A) Representative images of MDC staining BV2 cells were harvested by fluorescence microscope. B) The autophagy related proteins were evaluated by western blotting.
Figure 3

DHM pretreatment reversed LPS induced impaired autophagy and inhibited NLRP3 inflammasome activation in BV2 cells. A) Representative images of MDC staining BV2 cells were harvested by fluorescence microscope. B) The autophagy related proteins were evaluated by western blotting.

Figure 4
The effect of DHM on the expression of NLRP3 inflammasome, Iba-1 and mature IL-1β and IL18 proteins in the hippocampus. All data were expressed as M ± SD (n = 3/group). ###p < 0.001 relative to the SAMP8 group.

**Figure 5**

The effect of CQ on NLRP3 inflammasome and autophagy associated proteins in the hippocampus of SAMP8 mice. A) The autophagy related proteins and Sirt1 were evaluated by western blotting. B) Representative images of hippocampus were harvested by transmission electron microscope. Triangles represent the lipofuscin, and black arrows represent autophagosomes. Autophagosomes were observed as double membrane vacuoles yellow triangles. The images are representative of 3–4 mice per group. Scale bar in the upper panels (SAMP8, D1, D2, D2Q) 5μm. C) The NLRP3 inflammasome and proinflammatory factors were determined by western blotting. All data were expressed as M ± SD (n = 3/group). ###p < 0.001 relative to the SAMP8 group. ***p < 0.001 relative to the D2 group.
Figure 6

The effect of DHM on microglia polarization in SAMP8 mice. A) The M2 phenotype markers including CD206, IL-4 and Arg1 were evaluated by western blotting. B) Pro-inflammation cytokines were determined by corresponding antibody. All data were expressed as M ± SD (n = 3/group). #p<0.05, ###p < 0.001 relative to the SAMP8 group. **p<0.01, *** p <0.001 relative to the D2 group.

Figure 7
DHM attenuated LPS-induced NLRP3 dependent Sirt1 signaling. The NLRP3 and Cleaved-Caspase1 proteins were determined by western blotting. All data were expressed as M ± SD (n = 3/group). ### p < 0.001 relative to the Control group. *** p < 0.001 relative to the LPS group, &&& p < 0.001 relative to the LPS + DHM group.

Figure 8

The effect of DHM and CQ on learning and memory capacity of SAMP8 mice and AD-like pathological proteins. The learning and memory capacity was assessed by MWM (n = 10 per group). A) Latency to platform of the mice during 1-4 days. B) Latency to platform on the 5th day. C) Number of crossing target platform. D) Swimming track. E) The representative Nissl staining F) The expression of APP, BACE1, p-tau, tau was analyzed by Western blotting. ## p < 0.01, ### p < 0.01 relative to the SAMP8 group; * p < 0.05, ** p < 0.01, ***p < 0.001 relative to the D2 group.