Food and Chemical Toxicology 132 (2019) 110698

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Rubrofusarin inhibits Aβ aggregation and ameliorates memory loss in an Aβ-induced Alzheimer's disease-like mouse model

Eunbi Cho[a,1], Mudan Cai[b,1], Huiyoung Kwon[a], Jieun Jeon[a], Minho Moon[c], Mira Jun[d,e], Young Choon Lee[a,e], Jee Hyun Yi[f], Jong Hoon Ryu[b,**], Dong Hyun Kim[a,e,*]

[a] Department of Medicinal Biotechnology, College of Health Sciences, Dong-A University, Busan, 49315, Republic of Korea
[b] Department of Oriental Pharmaceutical Science, College of Pharmacy, Kyung Hee University, Seoul, 02447, Republic of Korea
[c] Department of Biochemistry, College of Medicine, Konyang University, Daejeon, 35365, Republic of Korea
[d] Department of Food Science and Nutrition, College of Health Sciences, Dong-A University, Busan, 49315, Republic of Korea
[e] Institute of Convergence Bio-Health, Dong-A University, Busan, 49315, Republic of Korea
[f] Center for Synaptic Brain Dysfunctions, Institute for Basic Science, Daejeon, Republic of Korea

ARTICLE INFO

Keywords:
Rubrofusarin
Alzheimer's disease
Amyloid β aggregation

ABSTRACT

The misfolding and aggregation of amyloid β (Aβ) peptide is a common histopathologic characteristic in patients with Alzheimer's disease, so is considered to play an critical role. In the present study, we examined the effect of rubrofusarin, an ingredient of Cassiae semen, on Aβ aggregation and memory loss in an AD mouse model. Rubrofusarin inhibited Aβ aggregation in a concentration-dependent manner. Moreover, rubrofusarin dis-aggregated preformed Aβ fibrils in a concentration-dependent manner. Although aggregated Aβ induced memory loss, Aβ pre-incubated with rubrofusarin failed to induce memory loss. Moreover, rubrofusarin administration ameliorated Aβ aggregates-induced memory loss. Finally, rubrofusarin reduced glial fibrillary acidic protein or Iba-1-positive area, markers of neuroinflammation, in the hippocampus of Aβ-treated mice. These results suggest that rubrofusarin can decrease Aβ fibril formation and ameliorate memory loss in the AD mouse model.

1. Introduction

Age-related diseases are increasingly becoming a problem for the patients and the society as the older population in the world is growing. Alzheimer's disease is a prevalent type of neurodegenerative disorder that slowly but surely devastates human intellect, showing dementia. The symptoms of AD include cognitive and behavioral dysfunction that interfere in daily performance (Burns et al., 1994). The pathological accumulation of amyloid β (Aβ) plaques and neurofibrillary tangles leads to degeneration of neurons and their synapses in the brain (Meyer et al., 2008). However, despite intensive research, AD continues to be defined as an incurable disease.

AD can be investigated through cognitive impairment assessment including Loewenstein occupational therapy cognitive assessment, mini-mental state examination, and the global deterioration scale (Patterson et al., 1990). Additional diagnosis on AD has performed using imaging techniques, including positron emission tomography and magnetic resonance imaging, which detect local functional and structural changes in the brain based on the presence of Aβ protein, Tau protein, and blood-based biomarkers (Pietrzak et al., 2018). The current pharmacologic treatment strategies for AD involve acetylcholinesterase inhibition or antagonist of NMDA glutamate receptor (Cummings et al., 2014). These are being developed and used as useful drugs after FDA approval; donepezil, rivastigmine, galantamine and memantine, however, the approach is symptomatic and palliative, not preventing or delaying the onset of AD (Scarpini et al., 2003). Most neurodegenerative disorders are proteinopathies, a group of diseases in which certain proteins undergo structurally misfolding and consequently, become accumulated (Jellinger, 2010; Uversky, 2009). Therapeutic agents directed towards this abnormality are a fundamental remedy, but have not been developed yet.

Protein aggregation is a common pathological phenomenon of neurodegenerative disorders (Ross and Poirier, 2004). Aβ peptide, frequently mentioned as crucial in the pathology of AD, is derived from the proteolytic cleavage of amyloid precursor protein (Howard et al. 2012) by β-site amyloid precursor protein cleaving enzyme 1 (BACE1)

* Corresponding author. Department of Medicinal Biotechnology, College of Health Sciences, Dong-A University, Busan, 49315, Republic of Korea.
** Corresponding author.
E-mail addresses: jhryu63@khu.ac.kr (J.H. Ryu), mose79@dau.ac.kr (D.H. Kim).
[1] These authors equally contributed in this study.

https://doi.org/10.1016/j.fct.2019.110698
Received 24 May 2019; Received in revised form 19 July 2019; Accepted 23 July 2019
Available online 23 July 2019
0278-6915/

and γ-secretase (Selkoe, 1997). Aβ monomer is consistently generated by the intrinsic enzyme, but spontaneously aggregates in a β-pleated sheet structure (Struble et al., 2010). The transition of monomeric Aβ into oligomers and plaques is a critical step to induce neurotoxicity and extracellular deposition, and exacerbate oxidative stress, metabolic decline and cell death, leading to dementia (Hardy and Selkoe, 2002). However, according to many reports, the level of this aggregation could be controlled by various natural compounds such as epigallocatechin gallate, curcumin, and resveratrol (Porat et al., 2006). Hence, therapeutic effort has focused on inhibiting aggregation and disassembling of Aβ in the brain to prevent AD.

Rubrofusarin (naphthopyrones) is a major component of the seed of Cassiae semen (Hatano et al., 1999). This seed has been medicinally used in Chinese to improve vision and as a cardiotonic, hypolipidemic, and diuretic agent; thus, it is useful in diverse disease such as anemia, cardiac disorders, dermatitis, liver problems, herpes, and mycosis (Singh et al., 2013). Rubrofusarin has anti-cancer, anti-bacterial infection, and anti-oxidant potential (Jing et al., 2011), but there has been no report on its role in governing protein aggregation yet. This study aimed to test the potential of rubrofusarin to prevent and treat AD-related pathology in a Aβ-induced AD mouse model.

2. Materials and methods

2.1. Thioflavin T fluorescence assay

Thioflavin T (ThT) was purchased from Sigma (T3516) and soluble in DPBS. $A\beta_{1\text{-}42}$ was dissolved at 1 mM in DMSO and diluted in DPBS, rubrofusarin stock was diluted in DMSO and curcumin stock was diluted in DMSO as a positive control. To quantify the extent of Aβ aggregate, $A\beta_{1\text{-}42}$ was mixed a 1:1 vol ratio with rubrofusarin in black Eppendorf tubes and incubated for 24 h at 37 °C. Final concentrations were $A\beta_{1\text{-}42}$ (10 μM), rubrofusarin (0, 10, or 30 μM) with ThT (45 μM). 100 μl of the mixture was put into a well of a 96-well plate (black plate). The fluorescence level was measured using an Synergy™ HTX multi-mode microplate reader with excitation at 485 nm and emission at 528 nm and performed in triplicate.

To monitor disaggregation, $A\beta_{1\text{-}42}$ was pre-incubated for 24 h at 37 °C with shaking to generate aggregate. Preformed aggregate were mixed with rubrofusarin as above for an additional 20 h at 37 °C. The fluorescence level was measured as described in the previous section.

2.2. Animals

ICR mice (6 weeks old, male) were purchased from Daehan Biolink (Choongbook, Korea). Mice were housed in the designated animal room (light/dark cycle = 12 h, temperature = 23 ± 2 °C, humidity = 50 ± 10%). All animals had free access to water and food. Mice were used for experiments after 1 week of acclimation. All animal experiments were approved by Institutional Animal Care and Use Committee of Dong-A University (DIACUC-approve-16-30).

2.3. Drug treatment

To test whether rubrofusarin inhibited generating toxic $A\beta_{1\text{-}42}$ aggregates, we incubated $A\beta_{1\text{-}42}$ with rubrofusarin for 24 h at 37 °C with shaking. Then, the $A\beta_{1\text{-}42}$, which were pre-incubated with rubrofusarin was injected into the third ventricle (stereotaxic coordinates: AP, −2.00 mm; ML, 0 mm; DV, −2.00 mm) (Paxinos and Franklin, 2004) of mice to examine whether the $A\beta_{1\text{-}42}$, could induce memory impairments. Next, to test whether rubrofusarin ameliorates $A\beta_{1\text{-}42}$ aggregates-induced memory impairments, we incubated $A\beta_{1\text{-}42}$ alone for 24 h at 37 °C with shaking. Then, the $A\beta_{1\text{-}42}$ aggregate was injected into the third ventricle of mice and rubrofusarin was administered for 7 days from 1 h after the injection of $A\beta_{1\text{-}42}$ aggregates. Behavioral experiments were conducted 1 h after the rubrofusarin administration at

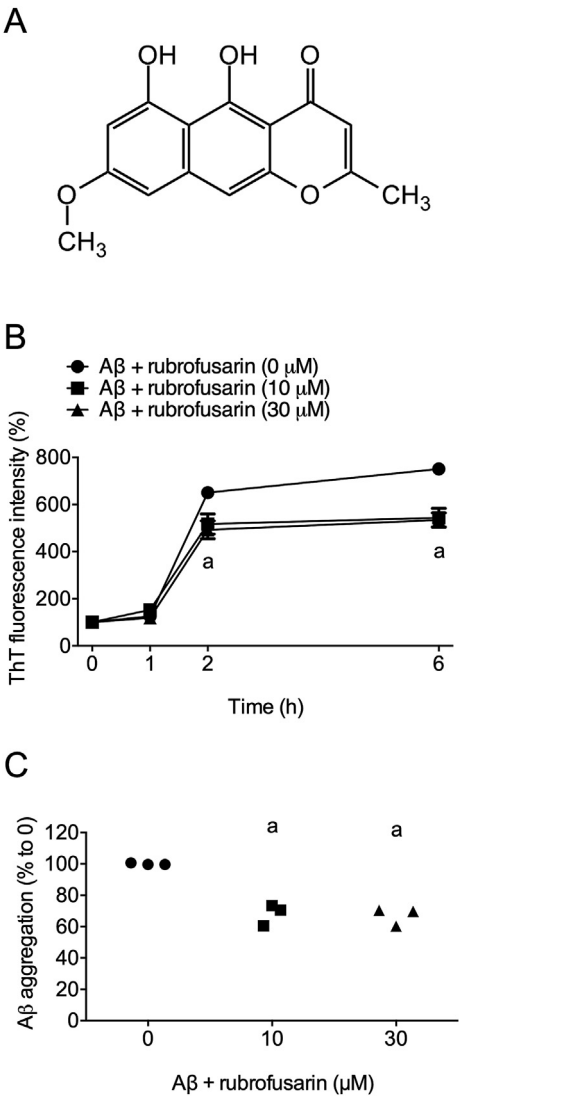

Fig. 1. Effect of rubrofusarin on Aβ aggregation. A. Structure of rubrofusarin. B and C. Monomeric Aβ was aggregated with or without rubrofusarin (0, 10 or 30 μM) for 6 h. B. ThT fluorescence intensity at each time point. Data represent mean ± SD. C. ThT fluorescence intensity at 6 h point. Data represents each raw value. a$P < 0.05$ vs. Aβ + rubrofusarin (0 μM) group.

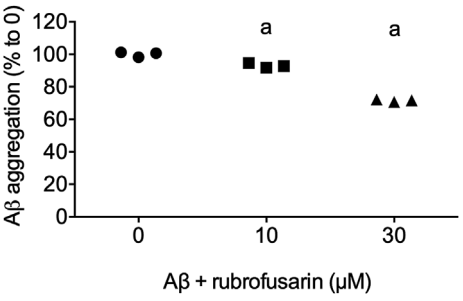

Fig. 2. Effect of rubrofusarin on preformed Aβ aggregate. Aβ was aggregated for 24 h and then, the Aβ aggregates was incubated with rubrofusarin (10 or 30 μM). Data represents each raw value. a$P < 0.05$ vs. Aβ + rubrofusarin (0 μM) group.

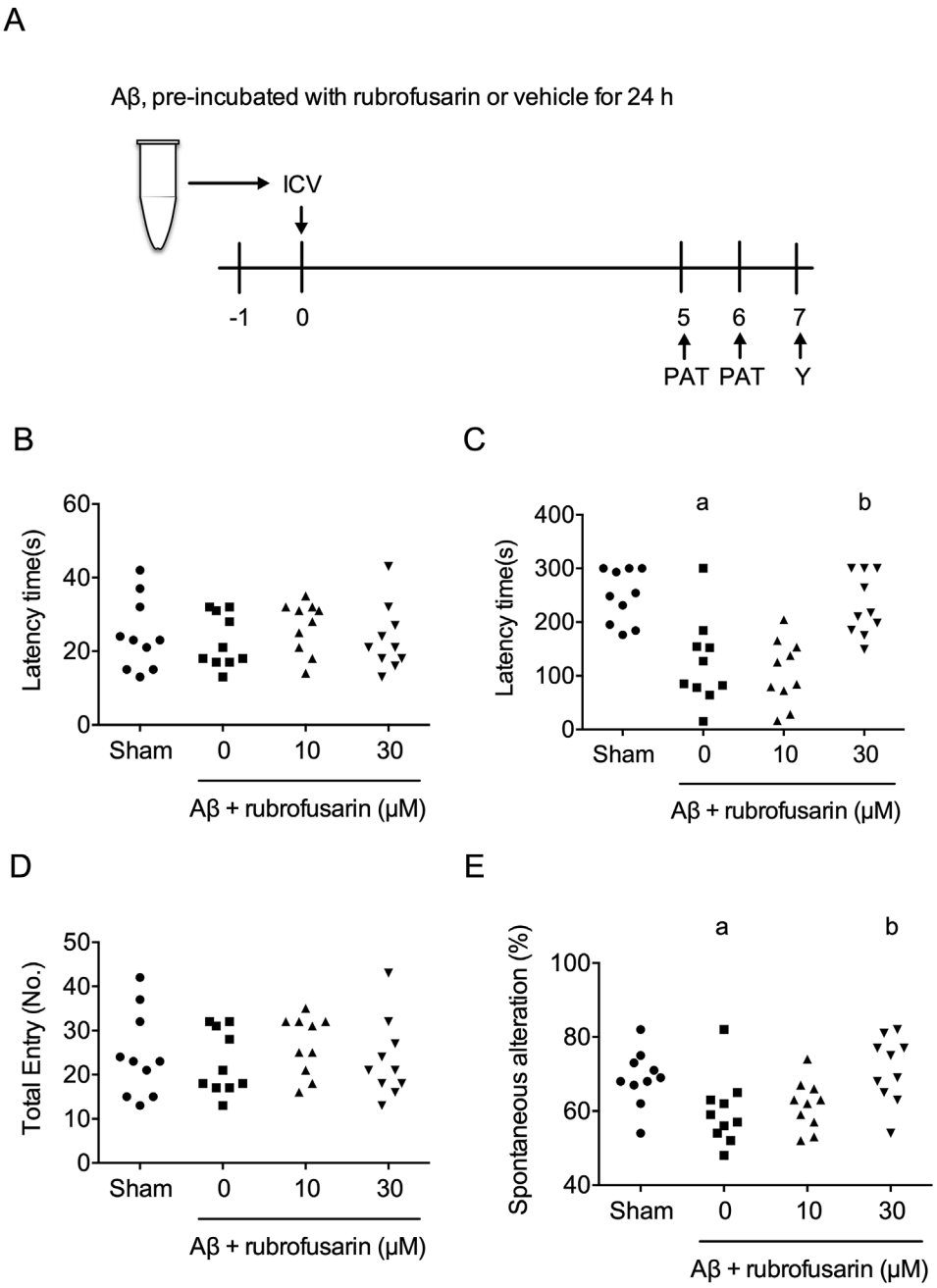

Fig. 3. Aβ incubated with rubrofusarin fails to induce memory deficit. A. Aβ was aggregated with rubrofusarin (10 or 30 μM) for 24 h and then, the aggregates were injected into third ventricle (ICV) of mice. Passive avoidance (PAT) and Y-maze (Y) tests were conducted 5 and 7 days after the Aβ injection, respectively. B. Latency time in training trial of passive avoidance test. C. Latency time in test trial of passive avoidance test. D. Total arm entry of Y-maze test. E. Spontaneous alternation of Y-maze test. Data represents each raw value. a$P < 0.05$ vs. sham. b$P < 0.05$ vs. Aβ + rubrofusarin (0 μM) group.

indicated day.

2.4. Passive avoidance test

Passive avoidance box is composed of two rooms; dark and illuminating rooms, which are separated with guillotine door. In a training session, mouse was located in the illuminating room and then guillotine door was opened 10 s later. When the mouse entered the dark room through the guillotine door, the door closed and an electric shock (0.5 mA for 3 s) was delivered through grid floor (training trial). The following day, mouse was re-introduced in illuminating room and guillotine door was opened 10 s later. Latency time to enter the dark room was measured for a period of 300 s (test trial).

2.5. Y-maze test

Y-maze test was conducted to test spontaneous alternation. Y-maze is composed of 3 black arms at an angle of 120° (40 × 5 × 15 cm). Mouse was located in one arm and it was monitored for a pattern of arm entrances for 8 min. When the mouse entered 3 different arms serially, we called it 1 actual alternation. Spontaneous alternation was calculated using the following formula: spontaneous alternation (%) = actual alternation/maximum alternation x 100 (maximum alternation = total entries – 2).

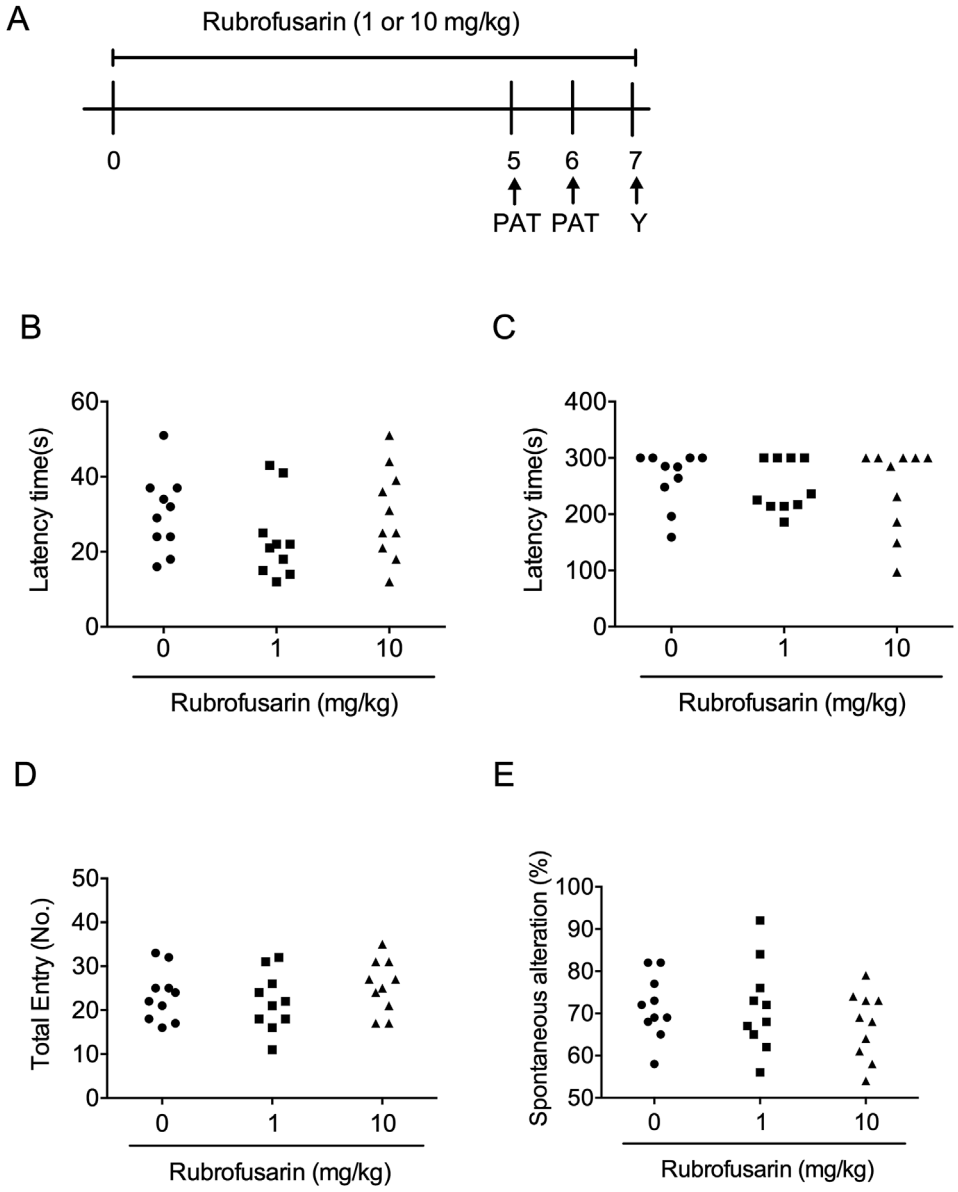

Fig. 4. Rubrofusarin did not affect learning and memory. A. Rubrofusarin (1 or 10 mg/kg, p.o.) was administered to mice for 7 days and the mice were examined in passive avoidance and Y-maze tests at 5 and 7-day, respectively. B. Latency time in training trial of passive avoidance test. C. Latency time in test trial of passive avoidance test. Data represent mean ± min to max. D. Total arm entry of Y-maze test. E. Spontaneous alternation of Y-maze test. Data represents each raw value. a$P < 0.05$ vs. sham. bP $<$ 0.05 vs. Aβ + rubrofusarin (0 mg/kg) group.

2.6. Immunohistochemistry

Mice were anesthetized using Zoletil 50® (10 mg/kg, i.m.) immediately after the Y-maze task and perfused transcardially with 100 mM phosphate buffer (pH 7.4) followed by ice-cold 4% paraformaldehyde. Brains were removed and postfixed in phosphate buffer (50 mM, pH 7.4) containing 4% paraformaldehyde overnight. The brains were immersed in a solution containing 30% sucrose in 50 mM phosphate-buffered saline (PBS) and stored at 4 °C until sectioning. Frozen brains were sectioned coronally on a cryostat at 30 μm, and sections including the hippocampal area (from −1.50 mm posterior to the bregma as defined in the mouse brain atlas) were stored in a storage solution at 4 °C.

Free-floating sections (thickness; 30 μm) were incubated for 24 h in PBS (4 °C) containing rat anti-iba-1 or goat anti-GFAP antibodies (1:1000 dilution), 0.3% Triton X-100 and 1.5% normal serum. The sections were incubated for 90 min with biotinylated secondary antibody (1:1000 dilution) followed by incubation with an avidin–biotin–peroxidase complex (1:100 dilution) for 1 h at room temperature. The sections were then treated with 0.02% 3, 3′-diaminobenzidine and 0.01% H_2O_2 for approximately 3 min followed by three washes with PBS. Finally, they were mounted on gelatin-coated slides, dehydrated in an ascending alcohol series, and cleared in xylene. Analysis (4 mice/group) was performed by an individual who was unaware of the treatment history. Immunopositive area was analyzed with ImageJ software.

2.7. Statistics

The results of all experiments were analyzed with one-way ANOVA followed by Turkey's test for multiple comparisons. Student's t-test was used to compare two groups. The values are expressed as the means ± S.E.M; $P < 0.05$ was considered significant.

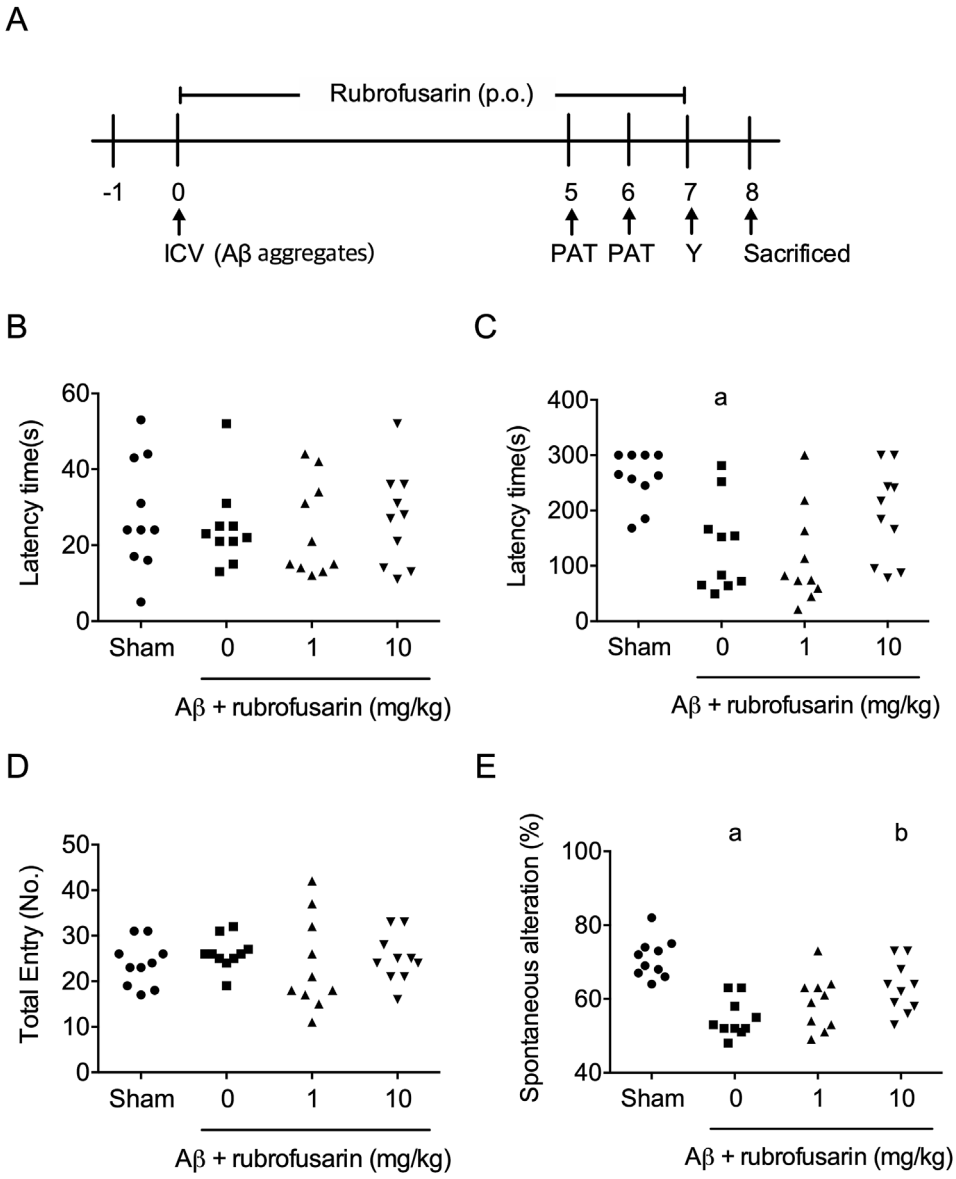

Fig. 5. Rubrofusarin ameliorates $A\beta_{1-42}$-induced memory deficit. A. Aβ was aggregated for 24 h and then, the aggregates were injected into third ventricle (ICV) of mice. Rubrofusarin (1 or 10 mg/kg, p.o.) was administered once a day from 1 h after to 7 days after the Aβ injection. Passive avoidance (PAT) and Y-maze (Y) tests were conducted 5 and 7 days after the Aβ injection, respectively. Mice were sacrificed at 8 days after the Aβ injection for immunohistochemistry. B. Latency time in training trial of passive avoidance test. C. Latency time in test trial of passive avoidance test. Data represent mean ± min to max. D. Total arm entry of Y-maze test. E. Spontaneous alternation of Y-maze test. Data represents each raw value. a$P < 0.05$ vs. sham. b$P < 0.05$ vs. Aβ + rubrofusarin (0 mg/kg) group.

3. Results

3.1. Rubrofusarin inhibits $A\beta_{1-42}$ aggregation

To find out the effect of rubrofusarin (Fig. 1A) on amyloid β (Aβ) aggregation in vitro, we measured the aggregation level using a thioflavin T (ThT) fluorescence assay. Aβ was co-incubated with rubrofusarin (0, 10, 30 μM) for 6 h. Rubrofusarin (10 or 30 μM) inhibited the increase in ThT fluorescence (Fig. 1B) and the difference was significant at 6 h ($F_{2,6} = 40.59$, $P < 0.05$, Fig. 1C). This result suggests that rubrofusarin could inhibit Aβ aggregation.

3.2. Rubrofusarin disassembles preformed $A\beta_{1-42}$ aggregate

Following the identification of the anti-aggregation effect of rubrofusarin, we measured the aggregation level using a ThT fluorescence assay to analyze the effect of rubrofusarin on dis-aggregation of preformed Aβ fibril. Aβ aggregate were prepared by pre-incubation at 37 °C for 24 h. Aβ fibril was co-incubated with rubrofusarin (0, 10, 30 μM) for further 24 h. Rubrofusarin significantly reduced ThT fluorescence intensity compared with the vehicle; DMSO ($F_{2,6} = 361.8$, $P < 0.05$, Fig. 2). This result suggests that rubrofusarin could disassemble Aβ fibril.

3.3. $A\beta_{1-42}$, which was pre-incubated with rubrofusarin, fails to induce memory loss

Because rubrofusarin inhibited $A\beta_{1-42}$ aggregation, we hypothesized that $A\beta_{1-42}$ failed to form toxic aggregates and failed to induced memory deficit when incubated with rubrofusarin. To investigate whether Aβ, which was pre-incubated with rubrofusarin, induced memory deficit, we incubated Aβ with rubrofusarin or vehicle for 24 h before the injection and injected this into the third ventricle of the mice. The passive avoidance and Y-maze tests were then performed (Fig. 3A). In the training trial of passive avoidance test, there was no significant effect between the groups in latency time ($F_{3,36} = 0.455$, $P > 0.05$, n = 10/group, Fig. 3B). In the test trial, Aβ, which was incubated with vehicle, significantly reduced latency time ($F_{3,36} = 13.22$, $P < 0.05$, n = 10/group, Fig. 3C); however, Aβ, which was incubated with rubrofusarin (30 μM), showed no effect in latency time ($P > 0.05$, Fig. 3C). In the Y-maze test, there was no significant effect between groups in total arm entry ($F_{3,36} = 0.462$, $P > 0.05$, n = 10/group, Fig. 3D). Aβ, which was incubated with vehicle, significantly reduced spontaneous alternation ($F_{3,36} = 4.512$, $P < 0.05$, n = 10/group,

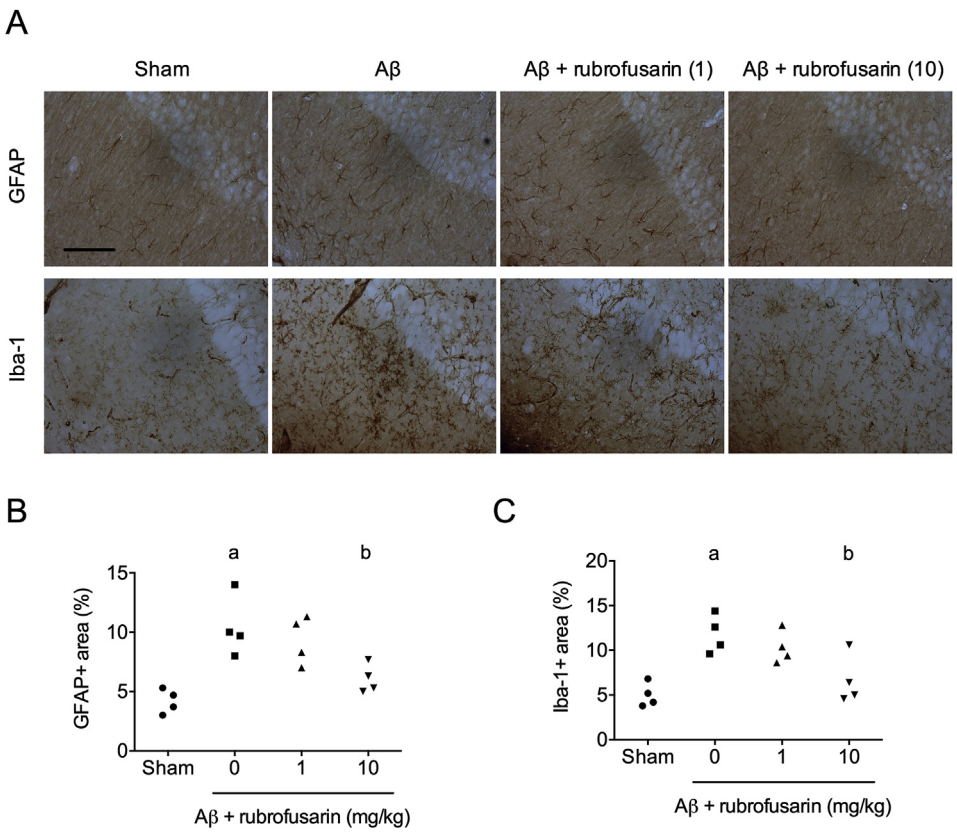

Fig. 6. Rubrofusarin ameliorates $A\beta_{1-42}$-induced brain inflammation. A. Photomicrograph of GFAP- and Iba-1-positive cells in the hippocampus. B. Quantitative analysis of GFAP-positive area in the hippocampus. C. Quantitative analysis of Iba-1-positive area in the hippocampus. Data represents each raw value. a$P < 0.05$ vs. sham. b$P < 0.05$ vs. Aβ + rubrofusarin (0 mg/kg) group.

Fig. 3E); however, Aβ, which was incubated with rubrofusarin, showed no effect in spontaneous alternation ($P > 0.05$, Fig. 3E). These results suggest that rubrofusarin inhibited formation of toxic Aβ aggregates.

3.4. Rubrofusarin ameliorates $A\beta_{1-42}$-induced memory loss

To investigate the effect of rubrofusarin in $A\beta_{1-42}$-induced memory impairments, we first investigated the effect of rubrofusarin, itself, on learning and memory. In these experiments, rubrofusarin failed to affect latency time in passive avoidance test and spontaneous alternation in Y-maze test (Fig. 4). Next, to test whether rubrofusarin ameliorates Aβ aggregates-induced memory loss, we examined rubrofusarin with a Aβ-induced memory loss model. Preformed Aβ aggregate was injected into the third ventricle and rubrofusarin was administered once daily for 7 days (Fig. 5A). In the training trial of passive avoidance test, there was no significant group effect ($F_{3,36} = 0.209$, $P > 0.05$, n = 10/group, Fig. 5B). However, in the test trial of passive avoidance test, the Aβ + vehicle group showed significant reduction in latency time compared with the control group ($P < 0.05$, Fig. 5C). The Aβ + rubrofusarin (10 mg/kg, p.o.) group showed significantly higher latency time than the Aβ + vehicle group ($F_{3,36} = 7.055$, $P < 0.05$, n = 10/group, Fig. 5C). In the Y-maze test, although there was no significant group effect in total arm entry ($F_{3,36} = 0.3026$, $P > 0.05$, n = 10/group, Fig. 5D), the Aβ + vehicle group showed a significant reduction in spontaneous alternation compared with the control group ($P < 0.05$, Fig. 5E). The Aβ + rubrofusarin (10 mg/kg, p.o.) group showed significantly higher spontaneous alternation than the Aβ + vehicle group ($F_{3,36} = 12.49$, $P < 0.05$, n = 10/group, Fig. 5E). These results suggest that rubrofusarin ameliorates memory loss induced by Aβ aggregate.

3.5. Rubrofusarin ameliorates $A\beta_{1-42}$-induced brain inflammation

To confirm the effect of rubrofusarin on Aβ aggregate-induced AD-like pathology, we observed glial activations induced by Aβ aggregate. Immediately after the Y-maze test, mice were sacrificed for immunohistochemistry (Fig. 6A). We investigated the neuroinflammatory markers iba-1 (microglia) and GFAP (astrocyte). In the iba-1 (microglia) immunohistochemistry, the Aβ + vehicle group showed significantly larger iba-1-immunopositive area in the hippocampus compared with the sham group. The Aβ + rubrofusarin (10 mg/kg, p.o.) group showed significantly smaller iba-1-immunopositive area cells in the hippocampus compared with the Aβ + vehicle group ($F_{3,12} = 10.19$, $P < 0.05$, n = 4/group, Fig. 6A and B). In the GFAP (astrocyte marker) immunohistochemistry, the Aβ + vehicle group showed significantly larger GFAP-immunopositive area compared with the control group. The Aβ + rubrofusarin (10 mg/kg, p.o.) group showed significantly smaller GFAP-immunopositive area compared with the Aβ + vehicle group ($F_{3,12} = 10.19$, $P < 0.05$, n = 4/group, Fig. 6A and C). These results suggest that rubrofusarin ameliorates Aβ aggregate-induced neuroinflammation.

4. Discussion

In this study, we found that rubrofusarin inhibited amyloid β (Aβ) aggregation. This was coherent with the result that Aβ, which was pre-incubated with rubrofusarin for 24 h, failed to induce memory loss, although Aβ, which was pre-incubated with vehicle, could induce memory loss in normal mice. This suggests that rubrofusarin blocked the formation of Aβ aggregate, which was not sufficient to induce memory loss. We also found that rubrofusarin disassembles pre-formed Aβ fibril. This was coherent with the result that Aβ-induced memory loss was blocked by rubrofusarin administration. This suggests that Aβ fibril was disassembled by rubrofusarin.

Misfolded Aβ assembly exhibited order-dependent increase in β-sheet content (Rzepecki et al., 2004). Because Aβ is one of the primary toxic elements in AD, Aβ targeting drug design is widely investigated. But still these attempts have failed to deliver clinical results, including

preventing Aβ processing, stabilizing monomers, inhibiting fibril growth, and disrupting amyloid assembly (Lu et al., 2019). Therefore, it may be a good approach to inhibit and inactivate amyloid aggregate using a competitive compound for β-sheet elongation at the terminal site. In fact, current research is focused on inhibition or disruption of aggregation by amyloid-β degrading enzymes, structure breakers and blockers (Poduslo et al., 1999; Salahuddin et al., 2016). In the present study, we found that rubrofusarin could be a breaker and blocker of Aβ aggregation.

Rubrofusarin is an active ingredient of the seed of Cassiae semen (Chinese senna, American sicklepod or sicklepod) (Singh et al., 2013). We previously reported that ethanol extract of the seed of *Cassia obtusifolia* ameliorated memory loss in various neurological diseases models including cerebral ischemia, Parkinson's disease and AD (Ju et al., 2010; Kim et al., 2009; Yi et al., 2016). Especially in AD model, *Cassia obtusifolia* inhibited neuroinflammation and blocked Aβ-induced dysfunction in Akt/glycogen synthase kinase-3β pathway (Yi et al., 2016). According to the results from the present studies, we can speculate that rubrofusarin in *Cassia obtusifolia* disassembled Aβ fibril and this could prevent the Aβ-induced neuroinflammation and abnormalization of molecular signals.

Neuroinflammation is a key pathological event in AD brain (Heneka et al., 2015). Because of the failure of various Aβ clearing therapies, including antibodies in clinical trials (Rosenblum, 2014), neuroinflammation raised up as a key area for developing treatment strategies for modulating AD. Although previous clinical studies dealing with anti-inflammatory drugs have failed (Stuchbury and Münch, 2005), neuroinflammation is still a major focus in AD therapy. In the present study, rubrofusarin inhibited neuroinflammation which is confirmed by the fact that rubrofusarin-treated group showed significantly lower Iba-1 and GFAP-positive area in the hippocampus. This could be induced by the effect of rubrofusarin on Aβ fibril. Rubrofusarin disassembled Aβ fibril and this failed to induced neuroinflammation. However, according to previous study, rubrofusarin seems to have an anti-inflammatory activity, itself (Paudel et al., 2018). Therefore, anti-inflammatory effect of rubrofusarin might be involved in its protective effect on Aβ fibril-induced neurotoxicity.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Conflicts of interest

There is no conflict of interest in this study.

Author's contributions

E.C. and M.M. conducted ThT assay and immunohistochemistry. J.H.Y and Y.C.L conducted animal behavioral experiments. H.K., J.J., and M.J. conducted immunohistochemistry. E.C. and J.H.Y wrote draft of manuscript. J.H.R and D.H.K planed and controlled all studies, and wrote manuscript.

Acknowledgements

This study was supported by the research fund of Dong-A University.

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