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Title Page

Icariin delays brain aging in senescence-accelerated mouse prone 8 (SAMP8) model via inhibiting autophagy

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Running title page

Running Title: Icariin delays brain aging

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Non-standard Abbreviations:

ICA: icariin; BCA: bicinchoninic acid; SAMP8: senescence accelerated mouse prone 8; SAMR1: senescence resistant series 1; MWM: Morris water maze; ANOVA: one-way analysis of variance; PBS: phosphate-buffered saline; SA-β-gal: Senescence-associated beta-galactosidase; LC3: microtubule-associated-protein-light-chain-3

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Abstract

Icariin (ICA), a major flavonoid extracted from the Chinese tonic herb Epimedium, exerts beneficial effects in a variety of age-dependent diseases, such as Alzheimer's disease (AD). However, the anti-aging mechanisms remain unclear. The senescence-accelerated mouse-prone 8 (SAMP8) model has been used to study age-related neurodegenerative changes associated with aging and the pathogenesis of AD. Hence, the current study was designed to examine the effect of ICA on age-related cognitive decline in SAMP8 mice and explore the role of autophagy in the ICA-mediated neuroprotection. SAMP8 mice were administered with ICA starting at 5 months of age, and the treatment lasted for 3 consecutive months. Morris water maze was used to evaluate cognitive function. The SA-β-gal staining was utilized to determine the number of senescence cells. The neuronal morphological changes were examined via Nissl staining. The hippocampal neuronal ultrastructure was examined by transmission electron microscopy. The expression of autophagy protein was examined by Western blot. ICA treated SAMP8 mice exhibited a robust improvement in spatial learning and memory function. Meanwhile, ICA reduced the number of senescence cells in the brain of SAMP8 mice, inhibited neuronal loss and reversed neuronal structural changes in the hippocampus of SAMP8 mice. Moreover, ICA treatment also decreased formation of autophagosomes in the hippocampus of SAMP8 mice, and

reduced the expression of autophagy-related proteins LC3-II and p62. These results demonstrate that ICA possesses the ability to delay brain aging in SAMP8 mice, and the mechanisms are possibly mediated through regulation of autophagy.

Introduction

Aging, a time-related deterioration of physiological functions, occurs in all of the organism's cell, tissue, and organs, leading to adverse changes, such as skin pigmentation, skin wrinkling and organ senescence (López-Otín et al., 2013). Aging itself cannot be considered as a disease, but aging is the greatest risk factor for the functional decline of most organs (Barzilai et al., 2018). In particular, aging is accompanied by a variety of senile diseases, such as dementia (Deak et al., 2016). With the development of social economy and the improvement of medical and sanitary conditions, the elderly population is steadily on the increase. Demographic data shows that 26.9% of the total population of the Chinese will be over 65 years old by 2050, China will be one of the countries in the world with the highest percentage of aged people (Fang et al., 2015). This will inevitably give rise to a host of socio-economic challenges. Accordingly, there is a scientific urgency to study the mechanisms of aging and develop effective anti-aging drugs to prevent age-related diseases. This is critical for nations such as China which is experiencing rapid growth in the aging population.

In the course of aging, the brain is probably the most vulnerable organ in the process because of high demand of oxygen, limited ability to regenerate and low endogenous antioxidant capacity (Mecocci et al., 2018). Brain aging is accompanied by many pathological changes and

behavior abnormalities, such as the decline in cognitive function, brain atrophy, lipofuscinosis, beta amyloid deposition and the loss of neurons, etc.(Serrano-Pozo et al., 2011). However, the mechanism of aging still remains elusive. Studies have suggested that autophagy is closely related to aging (Simonsen et al., 2008). Autophagy is a self-degradative process that cytoplasmic contents such as long-lived proteins, pathogen and damaged organelles are degraded by lysosomes (De Rechter et al., 2016). Autophagy thus plays a critical role in the prevention of neurodegenerative disorders, and dysfunction of autophagy affects cellular senescence and many other cellular signaling (Kang and Elledge, 2016). In addition, it has been demonstrated that autophagy plays an important role in the process of aging in the senescence accelerated mouse prone 8 (SAMP8) model (Wang et al., 2017b).

SAMP8, an animal model of aging, is an ideal model to study brain aging and dementia, and has been proposed as a mammal model to study rapid aging (Dang et al., 2018). SAMP8 mice present not only with Alzheimer disease features, such as Aβ deposition and excessive phosphorylation of tau protein, but also have similar characteristics of aged human, such as hair loss, shorter lifespan, reduced physical activity and lordosis (Akiguchi et al., 2017; Manich et al., 2011). Thus, SAMP8 and the senescence-accelerated mouse resistant 1 (SAMR1) mice of the same genetic background are used to study senility. In recent years,

several feasible strategies such as melatonin and caloric restriction have been studied to delay aging in SAMP8 mice, but they all have drawbacks (Cuesta et al., 2013; Garcia-Matas et al., 2015). Moreover, extracts from rosemary and sage, as well as antioxidants such as fish oil and alpha lipoic acid have also been shown to delay aging in SAMP8 mice (Tsuduki et al., 2011; Farr et al., 2012, 2016). With rich herbal medicine resources in China, we are searching for alternative approaches. In the present study, we employed SAMP8 and age-matched SAMR1 mice to study the beneficial effects of Icariin (ICA).

ICA, a major flavonoid extracted from the Chinese tonic herb Epimedium, exerts beneficial effects in a multitude of age-dependent disease states, including bone loss, cancer, cardiovascular disease, and neurodegenerative disorders (Li et al., 2015a). We and other groups have found ICA has anti-inflammatory, anti-oxidant, anti-angiogenesis and immunomodulation effects (Chen et al., 2014a; Li et al., 2015b; Wang et al., 2017a). Additionally, our previous study indicated that ICA improved spatial learning and memory abilities in lipopolysaccharide (LPS)-induced cognitive dysfunction through the inhibition of hippocampus IL-1β and cyclooxygenase-2 (COX-2) expressions (Guo et al., 2010). It has also been reported that ICA improves cognitive impairments in SAMP8 mice via increasing monoamines levels, inhibiting oxidative damage and decreasing acetylcholinesterase activity

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(He et al., 2010). In addition, ICA can protect PC12 cells from oxygen-glucose deprivation and reperfusion induced autophagy (Mo et al., 2016). However, whether the role of ICA in delaying aging in SAMP8 is related to the regulation of autophagy is unknown. Therefore, in the present study, we aimed to assess the preventative effects of ICA on SAMP8 mice, focusing on autophagy.

Materials and methods

Materials and Animals

Icariin (ICA, purity > 98%) was supplied from Nanjing Zelang Medical Technology Co., Ltd (Nanjing, China). All reagents were reagent grade and commercially available.

Male SAMP8 and SAMR1 mice were obtained from Medical Department of Beijing University with infection and virus free (Certificate No.SCXK2016-0010). All mice were maintained in Specific Pathogen Free (SPF) facilities of Zunyi Medical University (Certificate No.: SYXK 2014-003). Our SPF facilities were tested once a year by Chongqing Laboratory Animal Quality Testing Center. Mice were housed individually in the plastic cage with free access to food and water in a temperature- and humidity-controlled environment under a 12 h light/dark cycle. All animal experiments were strictly carried out in accordance with NIH guidelines for the Care and Use of Laboratory Animals (NIH Publications No.80-23, revised 1996), and the study protocol was approved by the Animal Use and Care Committee of Zunyi Medical University.

Experimental design and treatment

SAMP8 and age-matched SAMR1 mice were randomly divided into 5 groups: SAMR1 group, SAMP8 group, and SAMP8 group plus ICA groups (SAMP8 receiving 20, 40, or 80 mg/kg ICA). Mice were oral

administered once daily with normal saline (NS) or three doses of ICA, starting at the age of 5 months, and treatment lasted for 3 consecutive months. The doses and the duration of the treatment were based on our prior publications (Li et al., 2015; Jin et al., 2016). Four mouse right brains were fixed for histology, left hippocampal tissues were dissected for transmission electron microscopy, and the remaining brains were flash frozen for biological analysis, including Western blot.

Morris water maze test

The Morris water maze (MWM) test was carried out to evaluate the impact of ICA on spatial learning and memory ability of mice, as described previously (Liu et al., 2018). The pool was divided into four quadrants, with a hidden platform located 1 cm below the water level in the center of the target quadrant. Before the experiment, all mice were allowed to swim freely for 120 s to adapt to the water maze. In this task, mice received four training sessions (one session/day) and a probe trial on the 5th day. Each session consisted of three trials with a 2 h interval. A trial began when the mice was placed in the water at one of the three starting positions (excluding the platform quadrant), facing the wall. The swimming time of each mouse from the start location to reach the submerged platform (escape latency) was recorded. If the animal did not succeed it was gently guided to the platform and left on it for 10 s, and the escape latency was recorded as 90 s. On the fifth day, the platform

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was removed and the spatial probe test was carried out. The time spent in the target was measured by a computer-based video tracking system (Taimeng Co., Chengdu, China).

SA-β-gal staining

Animals were sacrificed after the behavior tests, four mice of each group were perfused transcardially with 0.1 M phosphate-buffered saline (PBS) and fixative solution of pre-cooled 4% paraformaldehyde, and brains were removed and immersion fixed in 4% paraformaldehyde. Frozen sections of 5-10 micron thickness were made by using cryomicrotome (Thermo Scientific, USA) for senescence-associated β-galactosidase (SA-β-gal) staining (Noren Hooten and Evans, 2017). After sectioning, tissues were covered with 500 μL pre-cooled fixative solution for 15 min at room temperature. Then, rinsed two times with PBS, and incubated with β-galactosidase staining solution at 37 ℃ overnight in a dry incubator (no CO_2). The stained tissues were examined under a light microscope (KS300, Zeiss-Kontron, Germany). For quantification, we used the Image J open source software to perform digital slide image counts.

Nissl staining

Brains were fixed in 4% paraformaldehyde, embedded by paraffin, and cut into coronal sections of 5 μm thick for Nissl staining (Liu et al., 2015). In brief, the sections were deparaffinized in xylene and rehydrated

using gradual alcohol, treated with Nissl staining solution (Solarbio, Beijing, China) for 5 min, and then mounted with neutral balsam. The hippocampal CA1 region were examined under a light microscope (Leica Microsystems Ltd., Wetzlar, Germany) by investigators who were blinded to the experimental groups. The numbers of Nissl bodies were captured in the three fields of the CA1 region of the hippocampus. For quantification, we used the Image J open source software to analyze.

Electron microscopy

For the experiments with electron microscopy, animals randomly selected from each group were sacrificed and left hippocampal tissues were dissected, immediately put in 2.5% glutaraldehyde, and postfixed with 1% OsO4. Tissues were dehydrated in a gradient series of diluted ethanol. After dehydration, the samples were infiltrated with a mixture of propylene oxide and epoxy resin (volume ratio =1:1) at 70℃ overnight. Ultra-thin sections (about 50 mm) were cut, mounted on a copper grid, stained with uranyl acetate and lead citrate, and observed using a transmission electron microscope (H-7650, Hitachi, Japan).

Western blot

The hippocampus and cerebral cortex were sheared into small pieces, and the total protein was extracted using the RIPA lysis buffer. The supernatants were collected by centrifugation at 12,000 rpm at 4℃ for 20 min, and the protein concentration was measured using the BCA protein

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assay kit (Beyotime, China). The equal amount of protein (30 μg) was separated by 10% SDS-PAGE, and transferred to 0.22 μm PVDF membranes (Millipore Trading Co. Ltd). The membranes were blocked in 5% skim milk for 2 h at room temperature. The membranes were then incubated at 4℃ overnight with primary antibodies against p62 (1:1,000 dilution, Abcam, USA), LC3-II (1:1,000 dilution, Abcam, USA) and β-actin (1:2,000 dilution, Beyotime, China). After being washed, the PVDF membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. The membrane-bound secondary antibody was detected using ECL select kit (Beyotime, China) and visualized using Gel Imaging (Bio-Rad, USA).

Statistical analysis

All results were analyzed by SPSS 16.0 statistics software and values were expressed as mean ± SD. The escape latency in the MWM was analyzed with two-way analysis of variance (ANOVA) and the Bonferroni test, and other results were analyzed by a one-way ANOVA. $P < 0.05$ was considered statistically significant.

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Results

Effects of ICA on learning and memory function of SAMP8 and SAMR1 mice

A training trial lasted for 4 days, and the escape latency time was recorded. As shown in Figure 1B, from Day 2 to Day 4, the mice in SAMP8 group presented significantly prolonged escape latency than that of SAMR1 group ($P < 0.05$), indicating the impairment of cognitive performances representing in SAMP8 group. However, the cognitive impairment of SAMP8 was improved after ICA administration, especially at the high dose of ICA (80 mg/kg) from Day 3 to Day 4 ($P < 0.05$). On the fifth day, the time spent in the platform quadrant was recorded (Figure 1C). Results showed that SAMP8 mice spent less time in the target quadrant than the SAMR1 mice ($P < 0.05$). The administration of ICA reduced the spatial memory impairment in a dose-dependent manner, particularly in the high dose group ($P < 0.05$).

Effects of ICA on senescence-associated β-galactosidase activity in the brain of SAMP8 and SAMR1 mice

The SA-β-gal staining was utilized to evaluate the effects of ICA on the aging of brain tissue. The blue particles indicated SA-β-gal activity. As shown in Figure 2A, we show that the intensity of blue particles was increased in the brain region of SAMP8 mice, and SAMP8 group exhibited about two-fold higher intensity of blue particles than SAMR1

mice ($P < 0.05$). However, a reduction in the positive expression of blue particles was observed in ICA treatment groups ($P < 0.05$), except for the mice receiving 20 mg/kg ICA (Figure 2B). These results suggest that ICA is able to slow down cellular senescence in the brain of SAMP8 mice.

Effects of ICA on hippocampal neurons of SAMP8 and SAMR1 mice

It has been documented that aging may cause neuronal atrophy or loss in the brain (Padurariu et al., 2012). To determine whether there is a loss of neuronal cell or structural alteration in the hippocampus of SAMP8 mice, Nissl staining was conducted to localize the cell body of neurons in the hippocampus. As shown in Figure 3, the hippocampus CA1 region of SAMP8 group exhibited abnormal neurons and loss of Nissl bodies. The pyramidal layer of cells was significantly diminished with a marked reduction (79%) in Nissl bodies in the hippocampus CA1 region in SAMP8 group compared with SAMR1 mice. The treatment of ICA significantly inhibited neuronal loss and reversed neuronal structural changes in the hippocampus of SAMP8 mice ($P < 0.05$).

Effect of ICA on autophagosome formation in the hippocampal neurons of SAMP8 and SAMR1 mice

Defects in autophagic activity are involved in a variety of neurodegenerative disorders (Son et al., 2012). Therefore, the hippocampal neuronal ultrastructure was examined by transmission

electron microscopy. The observation revealed that compared with SAMR1, the hippocampal neurons of SAMP8 group brain harbored disorganized and swollen endoplasmic reticulum. In addition, more autophagosomes were observed in hippocampal neurons of SAMP8 mice (Figure 4A, 4a, 4B, 4b). After administration of ICA, the number of autophagosomes was significantly decreased, accompanied by organized endoplasmic reticulum present in hippocampal neurons (Figure 4C, 4c, 4D, 4d, 4E, 4e).

The expression of LC3-II and p62 in the hippocampus and cortex of SAMP8 and SAMR1 mice

To define a possible role of autophogosome formation in the aging of SAMP8 mice, the protein expressions of autophagic marker LC3-II and p62 in the hippocampus and cortex were examined by Western blot. As shown in Figure 5, there was a significant increase in the expression levels of LC3-II and p62 in hippocampus and cortex of SAMP8 group compared with SAMR1 mice ($P < 0.05$). After receiving three months of ICA treatment, the expression of LC3-II and p62 reduced ($P < 0.05$), indicating the inhibitory effect of ICA on autophagosome formations in SAMP8 mice.

Discussion

The present study revealed that ICA treatment effectively attenuated cognitive deficits, reduced the number of senescence cells, inhibited neuronal loss and improved neuronal structural changes in the brain of SAMP8 mice. Simultaneously, ICA treatment also decreased formation of autophagosomes in the hippocampus of SAMP8 mice, and reduced the expression of autophagy-related proteins LC3-II and p62. This study is among the first to demonstrate ICA regulation of autophagy in SAMP8 mice.

SAMP8 mice are an excellent brain aging model of learning and memory defects (Akiguchi et al., 2017; Morley et al., 2012). Synaptic loss and cognitive deficits were increased significantly in 8-month-old SAMP8 mice (Chen et al., 2014b). Consistent with these reports, our present study showed that the learning and memory were significantly impaired in 8-month-old SAMP8 compared with the control strain SARM1 mice. The cognitive impairments of SAMP8 mice were significantly improved after the administration of ICA for three months, especially at the dose of 80 mg/kg, in agreement with the literature (He et al., 2010). Additionally, SAMP8 mice showed increased anxiety-like behavior compared to SAMR1 mice (Meeker et al., 2013), and ICA has been shown to have anti-anxiety effects (Li et al., 2014 ; Xiao et al., 2016), which could also contribute to cognition improvement.

Cellular senescence plays an important role in brain aging, increased senescent cells were observed in the lateral subventricular, hippocampus and cortical regions of aging mice (Carnero et al., 2013; Geng et al., 2010; Shimabukuro et al., 2016). SA-β-gal is a hydrolase enzyme to catalyze the hydrolysis of β-galactosides into monosaccharides only in senescent cells, and is regarded as a biomarker of cellular senescence (Itahana et al., 2007). The SA-β-gal staining assay has been widely used as biological indicators for aging cells, and senescent cells are stained with blue precipitate in the cytoplasm (Zhu et al., 2014). Therefore, we further explored the effect of ICA on aging using SA-β-gal assay to stain senescent cells in the cortex. Our results clearly demonstrated that 8-month-old SAMP8 mice receiving ICA treatment exhibited significantly decreased SA-β-gal staining positive aging cells in the cortex compared with untreated SAMP8 group. These findings indicated that attenuation of senescent cells may contribute to the protective ability of ICA against brain aging.

The hippocampus is an important part of the limbic system in the brain, to be more precise, emotion and cognition, as well as aging, are all closely related to hippocampal functions (Simic et al., 1997). Particularly, the hippocampus is vital for spatial learning and memory, and age-related progressive neuronal damage in hippocampus can lead to cognitive impairment (Borgesius et al., 2011; Chen et al., 1998; Thong-asa et al.,

2013). In addition, most studies have demonstrated that hippocampus is very sensitive to aging and is the first affected organ in terms of morphology and physiology in the aging process (Bhatnagar et al., 1997; Onozuka et al., 2002; Watanabe et al., 2002; West et al., 1994). To obtain more insight into the mechanism of how ICA improves cognitive function and reverses the aging process in SAMP8 mice, we further examined the structure of neuronal cell in the hippocampal CA1 region. As shown in Figure 3B, the hippocampus CA1 region of SAMP8 mice exhibited abnormal neurons and loss of Nissl bodies. The electron miscroscopy revealed disorganized endoplasmic reticulum in the hippocampus of 8-month-old SAMP8 mice (Figure 4B, 4b). All these results further confirmed the damage and loss of neurons in the brain of aging SAMP8 mice. Because neuronal loss in the hippocampal CA1 sub-region has been identified as memory-associated neuropathological marker in both humans and animals, and loss of neurons in the CA1 area occurs as age advances (Markham et al., 2005; Hosseini-sharifabad and Esfandiari., 2015; Banji et al., 2015). Strikingly, all these lesions and loss of neurons were significantly reduced in SAMP8 mice receiving ICA treatment for three months.

Importantly, we demonstrated that an increased autophagosome formation in hippocampal neurons occurred in the 8-month-old SAMP8 mice, compared with age-matched SAMR1 mice, supporting the notion

that alterations in autophagy occur in the aging process of SAMP8 mice (Ma et al., 2011; Chen et al., 2014b). Autophagy is a self-degradation pathway to eliminate damaged organelles (Mathiassen et al., 2017). However, autophagy is a double-edged sword and over-induction of autophagy may cause neuronal cell death. Aging is characterized by abnormal aggregation of proteins in brain neurons, triggering the induction of autophagy (Tan et al., 2014). Previous studies also reported that autophagic vacuoles were observed in the hippocampus of AD patients (Nixon et al., 2005). In the present study, electron microscopy revealed aging-dependent increase in autophagosome formation in the hippocampus of SMAP8 mice, which was inhibited by ICA. Furthermore, we also examined LC3 and p62 changes in the hippocampus and cortex, and found that the LC3-II expression showed an increase in hippocampus and cortex of 8-month-old SAMP8 mice, consistent with previous findings (Chen et al., 2014b; Ma et al., 2011). After administration of ICA, the expression of LC3-II decreased. Besides, the protein expression of p62, a marker of autophagy, was increased in SAMP8 mice, and diminished after administration of ICA. These results suggest that ICA could increase autophagosome-lysosome fusion, thus reducing the autophagy flux, in agreement of anti-autophagy effects of ICA in the literature (Algandaby et al., 2017; Li et al., 2017).

In conclusion, ICA has beneficial effects on improving the learning and memory impairment, reducing the number of senescence cells, inhibiting neuronal loss and reversing neuronal structural changes in SAMP8 mice, through a mechanism that may be related to the regulation of autophagy.

Author Contributions

Participated in research design: Qin Wu, Yun-Yan Xu, Jing-Shan Shi.

Conducted experiments: Fa-Ju Chen, Qin Wu, Yun-Yan Xu.

Performed data analysis: Fa-Ju Chen, Bo Liu, Qin Wu, and Jing-Shan Shi.

Wrote or contributed to the writing of the manuscript: Fa-Ju Chen, Bo Liu, Jie Liu, Shao-Yu Zhou, and Jing-Shan Shi.

Fa-Ju Chen and Bo Liu contributed equally to this work.

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Footnotes to Title

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Figure Legends

Figure 1: Effects of ICA on Morris water maze performance deficits in SAMP8 mice. A. Schematic representation of the experimental design; B. The latencies were measured to assess the mouse learning and memory ability in 4 days training trials; C. The percentage of time in the target quadrant. Data are presented as means ± SD (n=8-12). $^{*}P < 0.05$ vs SAMR1, $^{\#}P < 0.05$ vs SAMP8.

Figure 2: Effects of ICA on SA-β-gal activity in brain tissue of SAMP8 and SAMR1 mice. A. Representative images of SA-β-gal staining (magnification 200×, scale bar=50 μm); B. Quantitation of senescent positive cells. Data were expressed as mean ± SD (n=4). $^{*}P < 0.05$ vs SAMR1，$^{\#}P < 0.05$ vs SAMP8.

Figure 3: Effect of ICA on hippocampal neurons of SAMP8 mice. A. Representative images showing Nissl bodies in the hippocampal CA1 (magnification 200×, scalebar=50 μm); B. Quantitation of pyramidal cells in the CA1 hippocampal region. The numbers of Nissl bodies were captured in the three fields of the CA1 region of the hippocampus. The data represent mean ± SD (n=4). $^{*}P < 0.01$ vs SAMR1; $^{\#}P < 0.01$ vs SAMP8.

Figure 4: Effect of ICA on autophagosome formation in hippocampal neurons. The arrows indicate autophagosomes. Pictures in the upper panel of each group represent magnification 15,000×, scalebar=1 μm; Pictures in the lower panel of each group represent magnification 30,000×, scalebar=2 μm.

Figure 5: Effect of ICA on the expression of LC3-II and p62 in hippocampus and cortex. A. Representative bands of protein expression in hippocampus; B. Representative bands of protein expression in cortex; C. Quantitation of LC3-II protein level in hippocampus; D. Quantitation of LC3-II protein level in cortex; E. Quantitation of p62 protein level in hippocampus; F. Quantitation of p62 protein level in cortex. The relative optical density was normalized to β-actin. The data were expressed as mean ± SD (n=4). $^{*}P < 0.05$ vs SAMR1; $^{\#}P < 0.05$ vs SAMP8.

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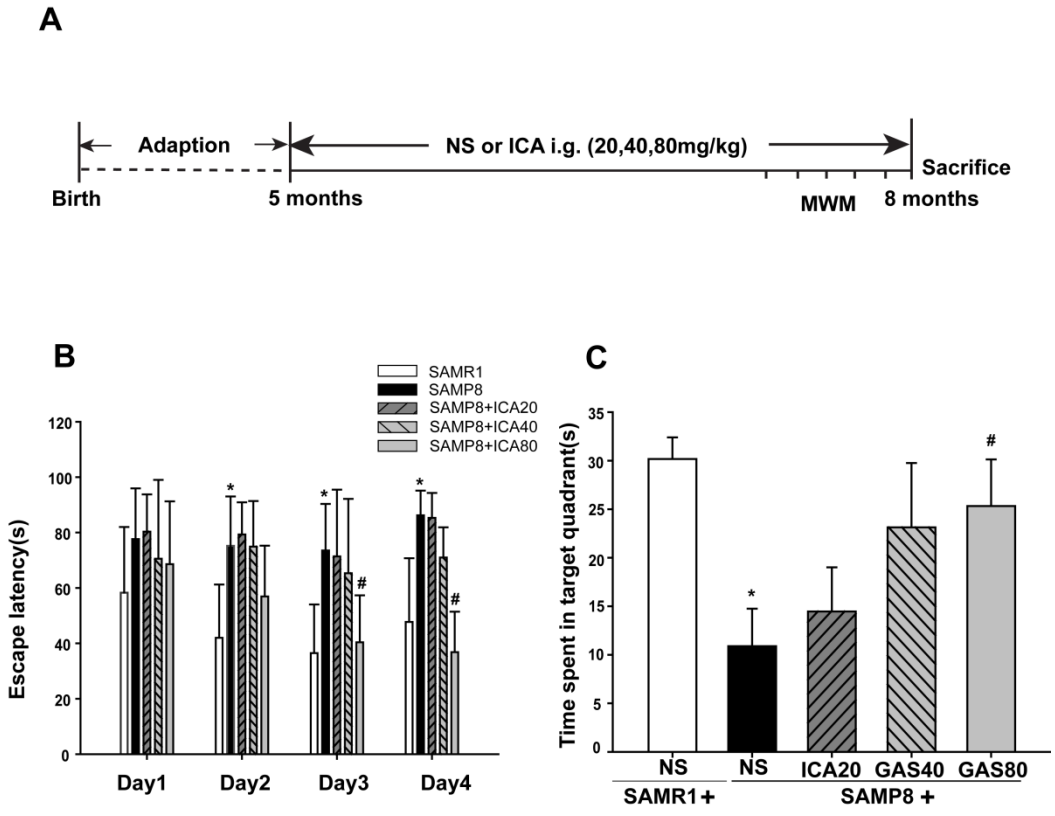

Figure 1

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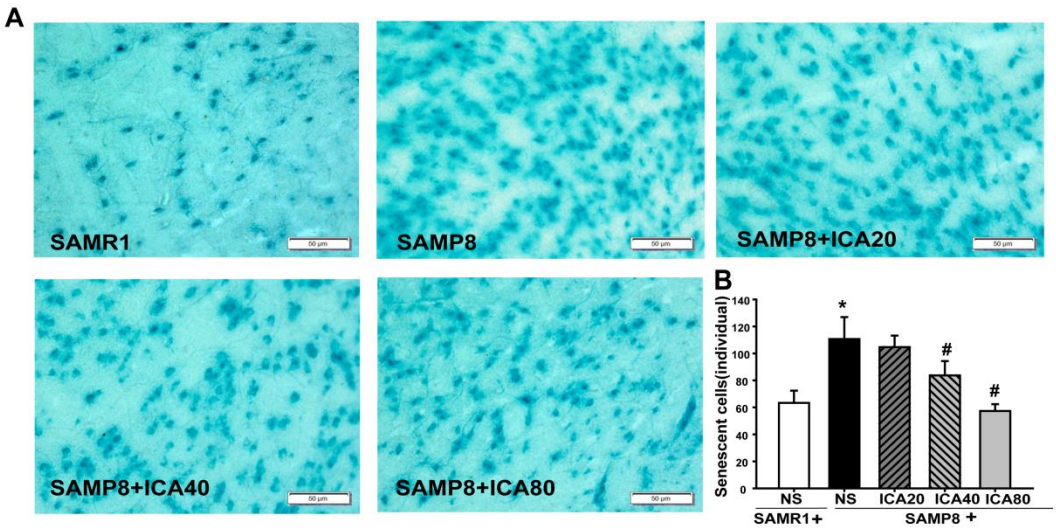

Figure 2

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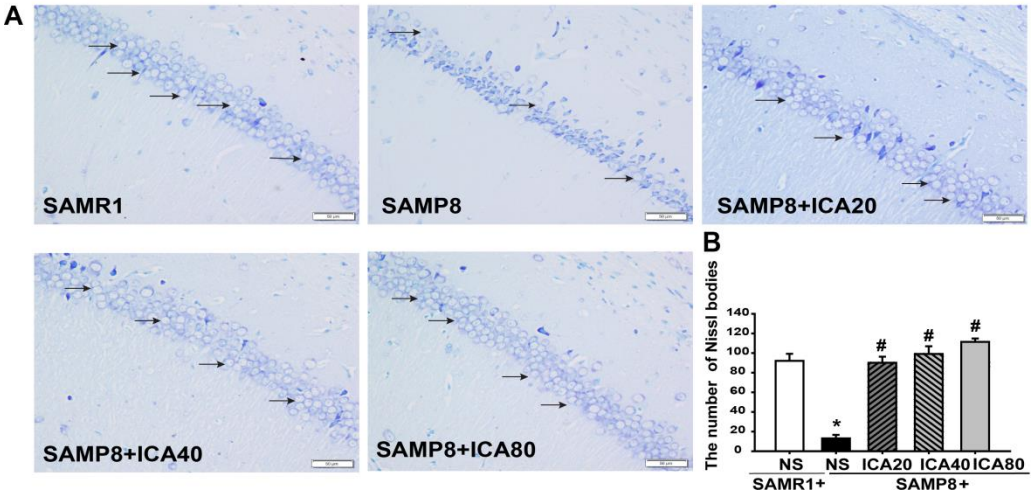

Figure 3

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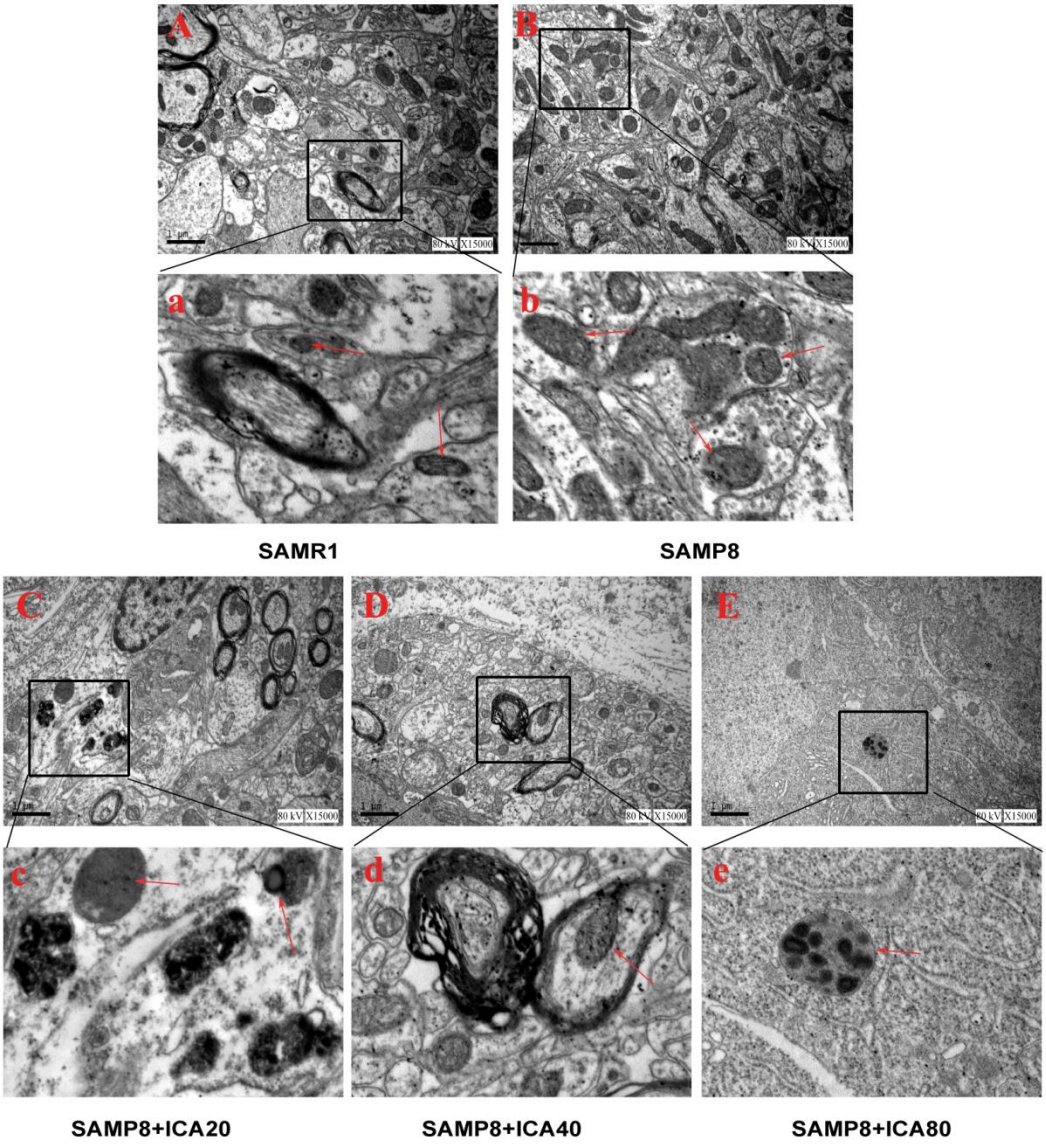

Figure 4