NAMPT-NAD$^+$ is involved in the senescence-delaying effects of saffron in aging mice

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Abstract. As the proportion of the elderly population grows rapidly, the senescence-delaying effects of Traditional Chinese Medicine is being investigated. The aim of the present study was to investigate the senescence-delaying effects of saffron in naturally aging mice. The active ingredients in an aqueous saffron extract were determined using high-performance liquid chromatography (HPLC). Mice were divided into saffron (8- and 16-months-old) and control groups (3-, 8-, and 16-months-old), and saffron extract was administered to the former groups for 8 weeks. The open field test and Barnes maze test were used to evaluate the locomotor activity, learning and memory function of the mice. The levels of inflammatory factors in the brain were determined by ELISA. In addition, the activities of acetylcholinesterase (AChE) and superoxide dismutase, and the contents of malondialdehyde and nicotinamide adenine dinucleotide (NAD$^+$) were detected by enzyme immunoassay, and the content of NAMPT was detected by ELISA, western blotting and reverse transcription-quantitative PCR. The cellular distribution of NAMPT and synaptic density were evaluated by immunofluorescence, and the pathological morphologies of the liver, skin, kidneys were observed by hematoxylin and eosin staining. HPLC revealed that the crocin and picrocrocin contents of the saffron extract were 19.56±0.14 and 12.00±0.13%, respectively. Saffron exhibited the potential to improve the learning and memory function in aging mice as it increased synaptic density and decreased AChE activity. Also, saffron ameliorated the pathological changes associated with organ aging, manifested by increasing the number of hepatocytes and the thickness of the skin, and preventing the aging-induced ballooning and bleeding in the kidneys. Furthermore, saffron increased the contents of NAMPT and NAD$^+$ in the brain and decreased the content of NAMPT in the serum. In addition, it changed the cellular distribution of NAMPT in aging mice, manifested as reduced NAMPT expression in microglia and astrocytes, and increased NAMPT expression in neurons. Saffron also decreased the contents of proinflammatory cytokines and oxidative stress factors in aging mice. Altogether, these findings indicate that saffron exerts senescence-delaying effects in naturally aging mice, which may be associated with the NAMPT-NAD$^+$ pathway.

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Introduction

As the proportion of the population who are elderly grows rapidly, the process of aging is of increasing concern to society, particularly as it is the primary risk factor for numerous chronic diseases and the primary driver of neurodegeneration (1). As such, it is essential to explore the mechanisms of aging and to discover novel anti-aging drugs. In recent years, a number of natural medicines have been found to delay senescence, including astragalus (2) and ginseng (3).

Saffron is the dry stigmas of the plant *Crocus sativus L.* of the family Iridaceae, which is widespread in several Asian countries. The active ingredients of saffron have been identified to include crocin, picrocrocin and safranal (4,5). Previous studies have reported that saffron or its active ingredients exert protective effects against Parkinson's disease (6), cerebral ischemia (7), tumors (8), and depression (9). In addition, saffron has also been shown to exert anti-aging effects, which manifest by inhibition of the loss of skin moisture and the exertion of anti-aging effects on the skin (10), by the reduction of β-secretase expression and inhibition GSK3β and ERK1/2 activity (11), and the alleviation of D-galactose-induced senescence in rats (12).

Nicotinamide phosphoribosyltransferase (NAMPT), also known as pre-B-cell colony enhancing factor or visfatin, is a key enzyme in the synthesis of nicotinamide adenine dinucleotide (NAD$^+$) (13), which exists both intracellularly and extracellularly (14). NAMPT is mainly expressed intracellularly in young mice and extracellularly in old mice. Regarding the cellular distribution in the brain, NAMPT is predominantly expressed in neurons and is absent from the microglia/astrocytes in young and healthy brains. However, it is mainly expressed in

the microglia/astrocytes upon aging (15). The increase in extracellular NAMPT levels and the change of cellular distribution can induce the secretion of inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-4 and oxidative stress factors from the microglia (14), which further promotes NAMPT secretion (16). NAD^+ is also an important substrate for redox processes and is closely associated with cellular metabolism, energy production and DNA repair (17). As such, it can be concluded that NAMPT-NAD^+ is involved in the senescence process (18), and may be a novel factor in the regulation of the aging process (19).

Our previous study showed that crocin ameliorated depressive-like behaviors induced by chronic restraint stress in mice by increasing the expression of NAMPT-NAD^+ (9). Saffron has also been shown to exert anti-inflammatory and anti-oxidative effects (20). However, it is unclear whether saffron exerts senescence-delaying effects by affecting the NAMPT-NAD^+ pathway and the subsequent inflammatory response. Therefore, in the present study, the senescence-delaying effects of saffron were explored to elucidate the possible mechanisms involving the NAMPT-NAD pathway.

Materials and methods

Preparation and detection of saffron aqueous extract. Saffron was purchased from Saffron Planting Base in Jiande, China. The saffron (6 g) was subjected to aqueous extraction five times under ultrasonication. The aqueous extract was then mixed and concentrated in a rotary evaporator under reduced pressure. Finally, it was lyophilized, yielding 3.7 g of aqueous extract (yield 61.6% w/w).

High-performance liquid chromatography (HPLC) was then carried out on a Waters 600E instrument, equipped with a Waters 2996 PDA detector (Waters GmbH). An Agilent C18 column (250x4.6 mm internal diameter; particle size, 5 μm; Agilent Technologies, Inc.) was used for separation. The HPLC conditions were as follows: Eluent A, double-distilled H_2O; eluent B, 95% acetonitrile; temperature at 25˚C; quantity of aqueous extract was 10 μl each time; gradient of A:B, from 87:13 at 0 min to 77:23 at 20 min; to 75:25 at 23 min; and to 50:50 at 45 min. The column was then equilibrated with an A:B ratio of 87:13 for 20 min at a flow rate of 1 ml/min. Peaks were assigned by comparison of their retention times with that of three reference compounds eluted in parallel with the same mobile phase, and by spiking the sample with reference compounds. The calibration curves of picrocrocin, crocin I and crocin II were $Y=15.932X + 0.00114$ ($R^2=0.9996$), $Y=39.867X - 54.766$ ($R^2=0.9993$) and $Y=49.66X - 50.675$ ($R^2=0.999$), respectively. The contents of the active ingredients were determined according to the corresponding calibration curves.

Animals and groups. A total of 40 female C57BL/6J mice (3 and 8 months old, 20-25 g; 16 months old, 25-30 g) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (certificate no. SCXK 2022-0004). All mice had food and water provided *ad libitum*, and were maintained at room temperature controlled at 22-24˚C and humidity at 50-60%, under a 12/12-h light/dark cycle. The C57BL/6J mice were randomly divided into the control groups (3-, 8- and 16-month-old), and the saffron groups (8- and 16-months-old), with 8 mice in each group. No mice died during this experiment. The Animal Health, Ethics and Research Committee of Hangzhou Medical College approved the study procedures.

Experimental protocol. The duration of the experiment was 63 days. Aqueous saffron extract was administered to the mice by oral gavage at a dose of 80 mg/kg once daily for 8 weeks. The mice in the control groups were given the same dose of double-distilled H_2O by oral gavage for 8 weeks. The open field test (OFT) and Barnes maze test (BMT) were performed on days 57, and 58-63, respectively, after the drug treatments (Fig. 1). Following the behavioral tests, on days 64, 3 mice in each group were anesthetized by the inhalation of isoflurane (4.5% for induction and 2% for maintenance). The mice were considered to be anesthetized when they breathed evenly, did not respond to their whiskers being touched and exhibited no toe-pinch pain reflex. They were then perfused with saline and 4% polyformaldehyde followed by rapid cervical dislocation. Tissue sections including brain, liver, kidneys and skin were then prepared to evaluate the cellular distribution of NAMPT and synaptic density by immunofluorescence, and organ-aging by hematoxylin and eosin (H&E) staining. The other 5 mice from each group were killed quickly by cervical dislocation to minimize suffering. No fluctuation in the chest, no visual response, and the eyelids becoming white confirmed the death of the mice. The brain tissues were quickly collected directly on ice. The brain was analyzed to detect the contents of NAMPT, NAD^+, malondialdehyde (MDA) and inflammatory factors, and the activity of superoxide dismutase (SOD) and acetylcholinesterase (AChE).

OFT. Briefly, the mice were habituated to the laboratory environment one day before the test. The next day, the mice were gently placed in the center of a chamber (40x40x40 cm; Shanghai Xinruan Information Technology Co., Ltd.). Each mouse was allowed to habituate to the chamber for 5 min, and then allowed to explore freely for 5 min while a video recording was made. The total distance traveled and the mean velocity of movement were measured.

BMT. Mice were placed in the center of a circular platform with 20 equally spaced holes, one of which was connected to a safe chamber (Shanghai Xinruan Information Technology Co., Ltd.). Aversive noise (65 dB) was used to encourage the mouse to locate the safe target. All mice were trained for 5 consecutive days with 4 min per trial, once daily. Tests were conducted on day 6, with only one chance to reach the target given. The number of times the mice explored each hole, and the latency to find the target hole were recorded.

Enzyme-linked immunosorbent assays (ELISAs). The brains collected on ice were rapidly dissected, and a homogenate was obtained by sonication. After the removal of particulates by centrifugation (1,450 x g, 4˚C, 20 min), the levels of TNF-α, IL-1β, IL-4 and IL-10 in the brain, and NAMPT in the brain and serum were measured using the corresponding ELISA kits (cat. nos. ml002095, ml301814, ml002149 and ml002285, respectively; Shanghai Enzyme-linked Biotechnology Co., Ltd.), in accordance with the manufacturer's instructions. The contents of TNF-α, IL-1β, IL-4, IL-10 and NAMPT were calculated by reference to the corresponding standard curve.

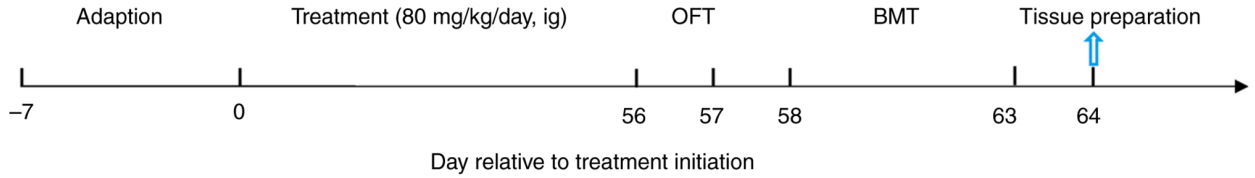

Figure 1. Experimental design. OFT, open field test; BMT, Barnes maze test; ig, intragastric.

Western blotting. Brains were dissected and homogenized in ice-cold RIPA lysis buffer (Beyotime Institute of Biotechnology). Then they were centrifuged with 8,700 x g at 4˚C for 10 min to obtain total proteins. After that each sample was quantitatively tested by Nano Drop, and loaded with the same total protein amount (50 mg). The proteins were separated by 10% SDS-PAGE, and transferred to PVDF membranes using an electrophoretic transfer system (Bio-Rad Laboratories, Inc.). Membranes were blocked against non-specific binding in 5% skimmed milk for 1 h at room temperature. The membranes were then incubated with anti-NAMPT (1:5,000; cat. no. A300-372A; Bethyl Laboratories, Inc.) or anti-GAPDH (1:1,000; cat. no. AF1186; Beyotime Institute of Biotechnology) primary antibody overnight at 4˚C. The membranes were washed by TBST (0.1% tween-20; Beyotime Institute of Biotechnology) and incubated with HRP-labeled goat anti-rabbit IgG (H+L) (1:200; cat. no. A0208; Beyotime Institute of Biotechnology for 1 h at room temperature. Then BeyoECL Moon (cat. no. P0018FS; Beyotime Institute of Biotechnology) was used for visualization. Finally, the results were analyzed using the Tanon 5200 Imaging System (Tanon Science and Technology Co., Ltd.).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the brain using an RNA extraction kit (cat. no. R6934-01; Omega Bio-Tek, Inc.). After measuring the purity and concentration of the RNA, reverse transcription to cDNA was performed using a Hifair® II 1st Strand cDNA Synthesis SuperMix for qPCR (gDNA digester plus) (cat. no. 11123ES60; Shanghai Yeasen Biotechnology Co., Ltd.) at 25˚C for 5 min; and 42˚C for 30 min; then at 85˚C for 5 min. qPCR amplification was then performed using the cDNA as the template by YeaRed Nucleic Acid Gel Stain (cat. no. 10202ES76; Shanghai Yeasen Biotechnology Co., Ltd.). The thermocycling conditions were as follows: Initial denaturation at 95˚C for 2 min; followed by 42 cycles of 95˚C for 10 sec and 55˚C for 30 sec. The relative expression of *Nampt* mRNA was calculated using the $2^{-\Delta\Delta Cq}$ method (21) with the following primers: NAMPT forward: 5'-GCAGAAGCCGAGTTCAACATC-3' and reverse: 5'-TTTTCACGGCATTCAAAGTAGGA-3'; GAPDH forward: 5'-AAGAAGGTGGTGAAGCAGG-3' and reverse: 5'-GAAGGTGGAAGAGTGGGAGT-3'.

Enzyme immunoassays. The enzymatic activity of SOD (cat. no. BC010-2) and AChE (cat. no. BC2025), and the contents of NAD^+ (cat. no. SH985W) and MDA (cat. no. BC003-2) in the brain were quantified using corresponding kits (Beijing Solarbio Science & Technology Co., Ltd.), according to the manufacturer's instructions. Absorbance values were subsequently determined at the appropriate wavelength.

Immunofluorescence and H&E staining. After the last behavioral test, mice were sacrificed and the brain, liver, kidneys and skin were removed following perfusion through the left ventricle with saline solution and 4% paraformaldehyde. The tissues were submerged in 4% paraformaldehyde for 24 h at 4˚C, and dehydrated with 30% sucrose solution for 2-3 days. Sections were then prepared with a frozen microtome. The 5% goat serum (cat. no. C0265; Beyotime Institute of Biotechnology) was used to block the tissue at 37˚C for 2 h. The thickness of liver and kidneys were 8 μm, skin sections were 6 μm and brain sections were 25 μm.

For immunofluorescence, the sections were incubated in anti-NAMPT (1:500; cat. no. A300-372A), anti-neuronal nuclei (NeuN; 1:100; cat. no. MAB377; Sigma-Aldrich; Merck KGaA), anti-glial fibrillary acidic protein (GFAP; 1:10; cat. no. BM2287; OriGene Technologies, Inc.), anti-ionized calcium-binding adapter molecule 1 (Iba-1; 1:100, cat. no. 019-19741; Wako Pure Chemical Industries, Ltd.), anti-postsynaptic density protein 95 (PSD95; 1:200; cat. no. 3450S; Cell Signaling Technology, Inc.) and anti-synaptophysin (1:200; cat. no. 9020S; Cell Signaling Technology, Inc.). After incubation with Cy3-labeled goat anti-rabbit IgG (H+L) (1:200, cat. no. A0516; Beyotime Institute of Biotechnology) and FITC-labeled goat anti-mouse IgG (H+L) (1:200, cat. no. A0568; Beyotime Institute of Biotechnology) for 2 h at room temperature, the sections were mounted with DAPI. Images of the sections were captured using confocal microscope, and the quantitative assessment was subsequently performed by Image J 1.0 software (National Institutes of Health).

All samples of the liver, kidneys and skin were stained with H&E at room temperature for 4 min, and to analyze the pathological changes under a light microscope. The thickness of the skin was measured at 6 randomly selected regions using Image J software (National Institutes of Health).

Statistical analysis. All data were analyzed using SPSS 25.0 software (IBM Corp.), and are presented as the mean ± SEM. One-way analysis of variance followed by LSD (homogeneity of variance) and Tamhane's T_2 (heterogeneity of variance) test were performed to analyze differences among groups. $P<0.05$ was considered to indicate a statistically significant result.

Results

Contents of active components in saffron. The HPLC results showed that the average content of the saffron active

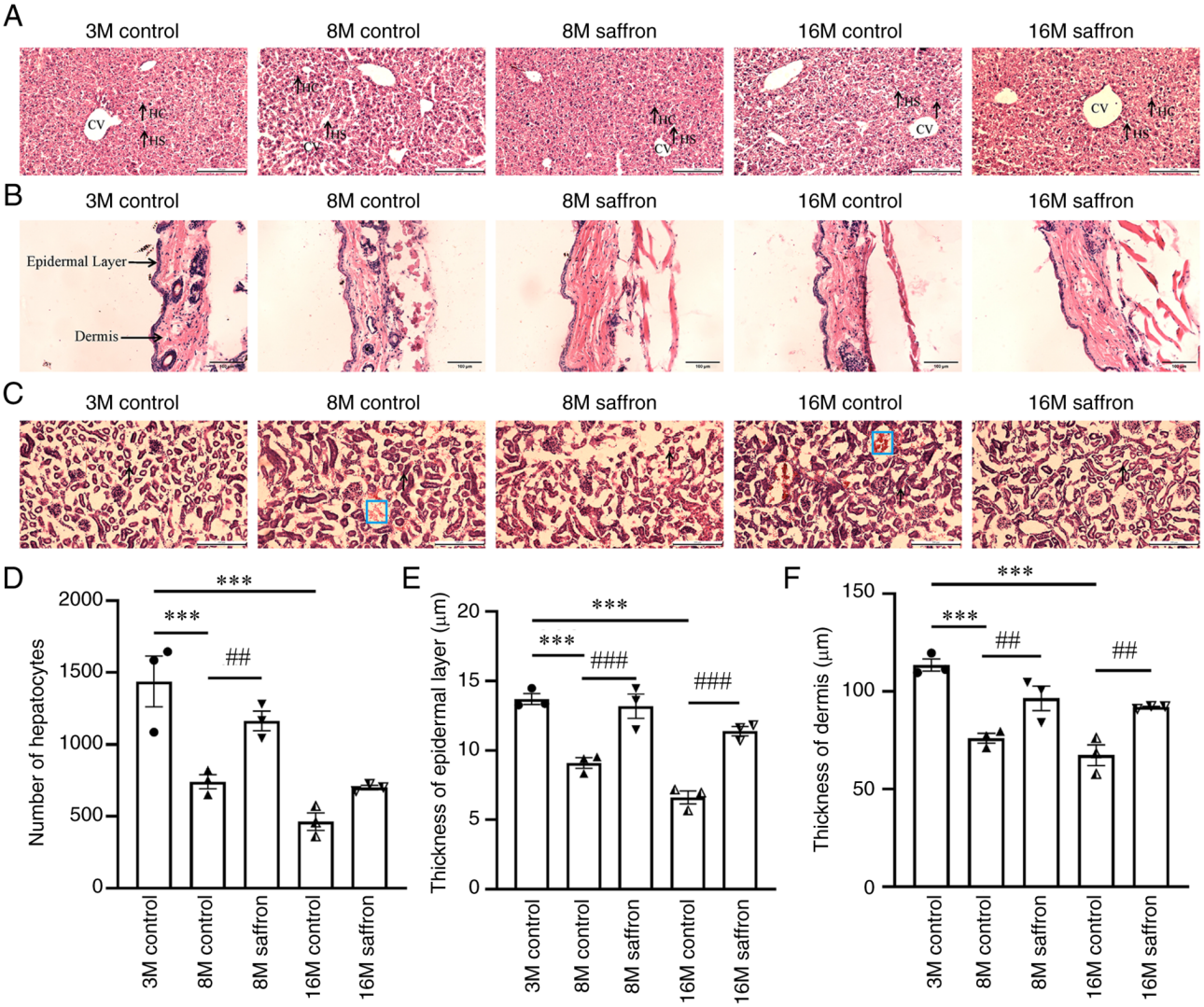

Figure 2. Effects of saffron on the pathological morphology of the liver, kidneys and skin in mice. Pathological morphology of the (A) liver (scale bar, 200 μm), (B) skin (scale bar, 100 μm) and (C) kidneys (scale bar, 200 μm) as revealed by hematoxylin and eosin staining (blue boxes indicated bleeding in the area). (D) Hepatocyte number, (E) thickness of the epidermal layer and (F) thickness of the dermis. Values are presented as the mean ± SEM. ***P<0.001 vs. the 3-month-old control group; $^{##}$P<0.01, $^{###}$P<0.001 vs. the age-matched control group. HC, hepatocyte; HS, hepatic sinusoid; CV, central vein; M, month.

components picrocrocin, crocin I and crocin II was 12.00±0.13, 10.80±0.27 and 8.77±0.19%, respectively (Table I and Fig. S1).

Effects of saffron on the pathological morphology of the liver, kidneys and skin in mice. Analysis of pathological morphology in the mice revealed fewer hepatocytes and more pyknosis in the liver tissue in the 8- and 16-month-old control mice than the 3-month-old control mice. Additionally, both 8- and 16-month-old groups were characterized by the irregular arrangement of hepatocytes with vacuolated cytoplasm. Shrunken central veins with blood congestion and irregular blood sinusoids were also detected. However, sections from saffron-treated mice exhibited almost normal hepatocyte nuclei, and an intact central vein (Fig. 2A). Further, the number of hepatocytes was 57.03% higher in the 8-month-old mice with saffron treatment compared with the 8-month-old control mice (P<0.01; Fig. 2D).

Fewer and thinner dermal collagen fibers, and the infiltration of dermal inflammatory cells were observed in the 8- and 16-month-old control groups (Fig. 2B), while the thickness of the epidermis and dermis were decreased compared with those in the 3-month-old control group (all P<0.001; Fig. 2E and F). In the 8- and 16-month-old mice, saffron treatment increased the thickness of the epidermis (both P<0.001; Fig. 2E) and dermis (both P<0.01; Fig. 2F) compared with those in the age-matched controls.

Increased hemorrhage and ballooning were observed in the kidneys of the 8- and 16-month-old control groups when compared with the 3-month-old control group, and saffron treatment ameliorated these changes (Fig. 2C).

Table I. Contents of the main components of saffron aqueous extract.

Variable	Picrocrocin	Crocin I	Crocin II
Average peak area	639.46	1,245.36	645.06
Average content, %	12.00±0.13	10.80±0.27	8.77±0.19

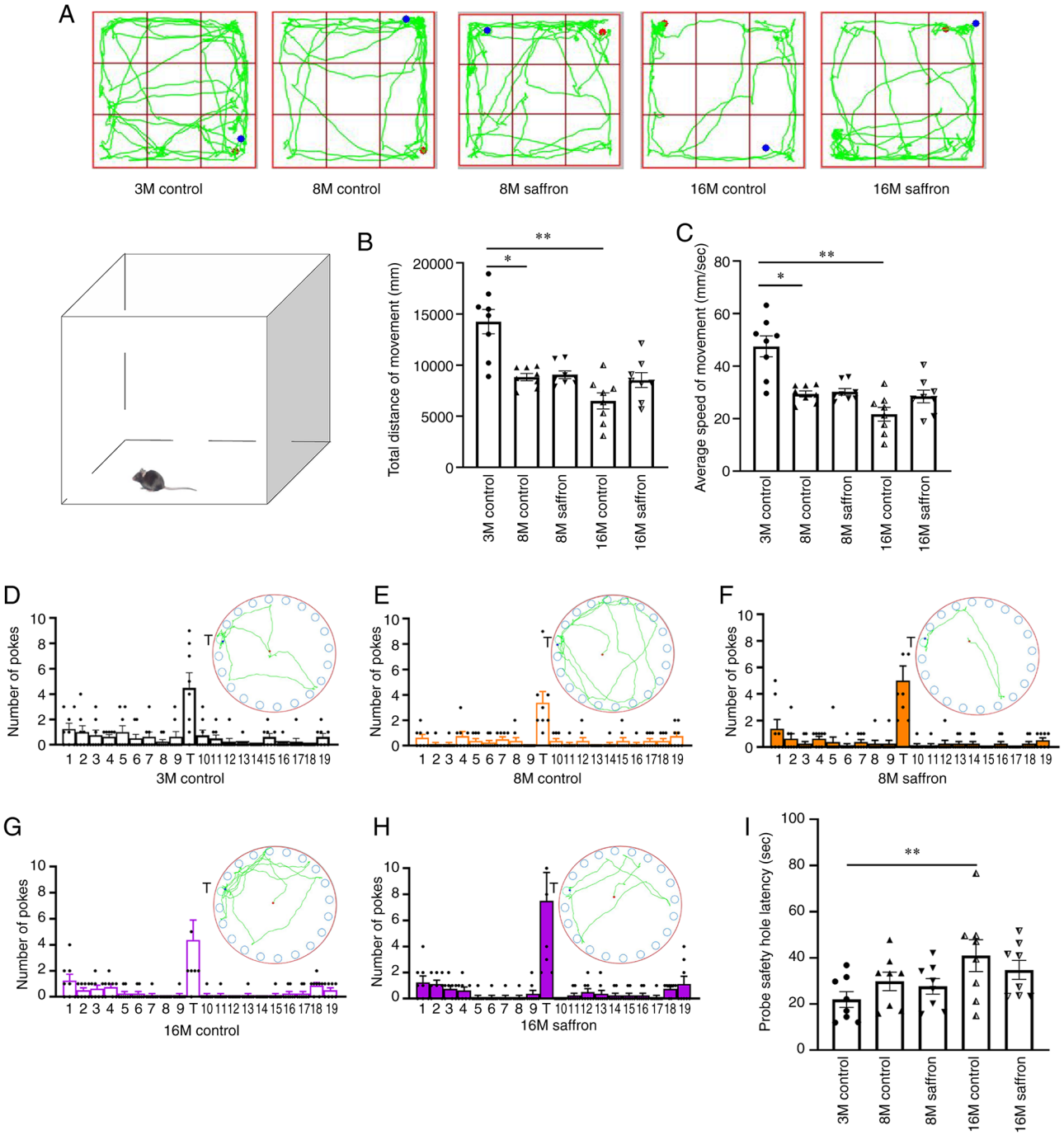

Figure 3. Effects of saffron on the OFT and BMT performance of mice. (A) Typical trajectory plots of different groups in the OFT. (B) Total distance travelled and (C) mean velocity of movement in the OFT. (D-H) Typical trajectory plots in the BMT and the number of explorations each hole in the (D) 3M control, (E) 8M control, (F) 8M saffron, (G) 16M control and (H) 16M saffron groups. (I) Probe safety hole latency for each group in the BMT. Values are presented as the mean ± SEM (n=8). *P<0.05 and **P<0.01 vs. the 3-month-old control group. OFT, open field test; BMT, Barnes maze test; M, month; T, target hole.

Effects of saffron on the behavior of the mice. In the OFT, the 8- and 16-month-old control mice had a shorter total traveled distance (P<0.05 and P<0.01, respectively) and slower mean velocity (P<0.05 and P<0.01, respectively) compared with the 3-month-old mice. However, no difference was detected between the same-aged saffron and control groups (Fig. 3A-C). Furthermore, no significant difference in the number of explorations of the target hole among groups was observed in the BMT (Fig. 3D-H). However, the probe safety hole latency was significantly increased in the 16-month-old control mice compared with 3-month-old mice in the BMT (P<0.01; Fig. 3I).

Effects of saffron on synaptic density and AChE activity in mice. PSD95 and synaptophysin were co-expressed to determine synaptic density (22). Compared with the synaptophysin density in the brains of the 3-month-old control group, that in the 8- and 16-month-old control groups decreased by 41.60 and 47.37%, respectively (both P<0.001). Following saffron treatment, synaptophysin density increased by 51.52% in the

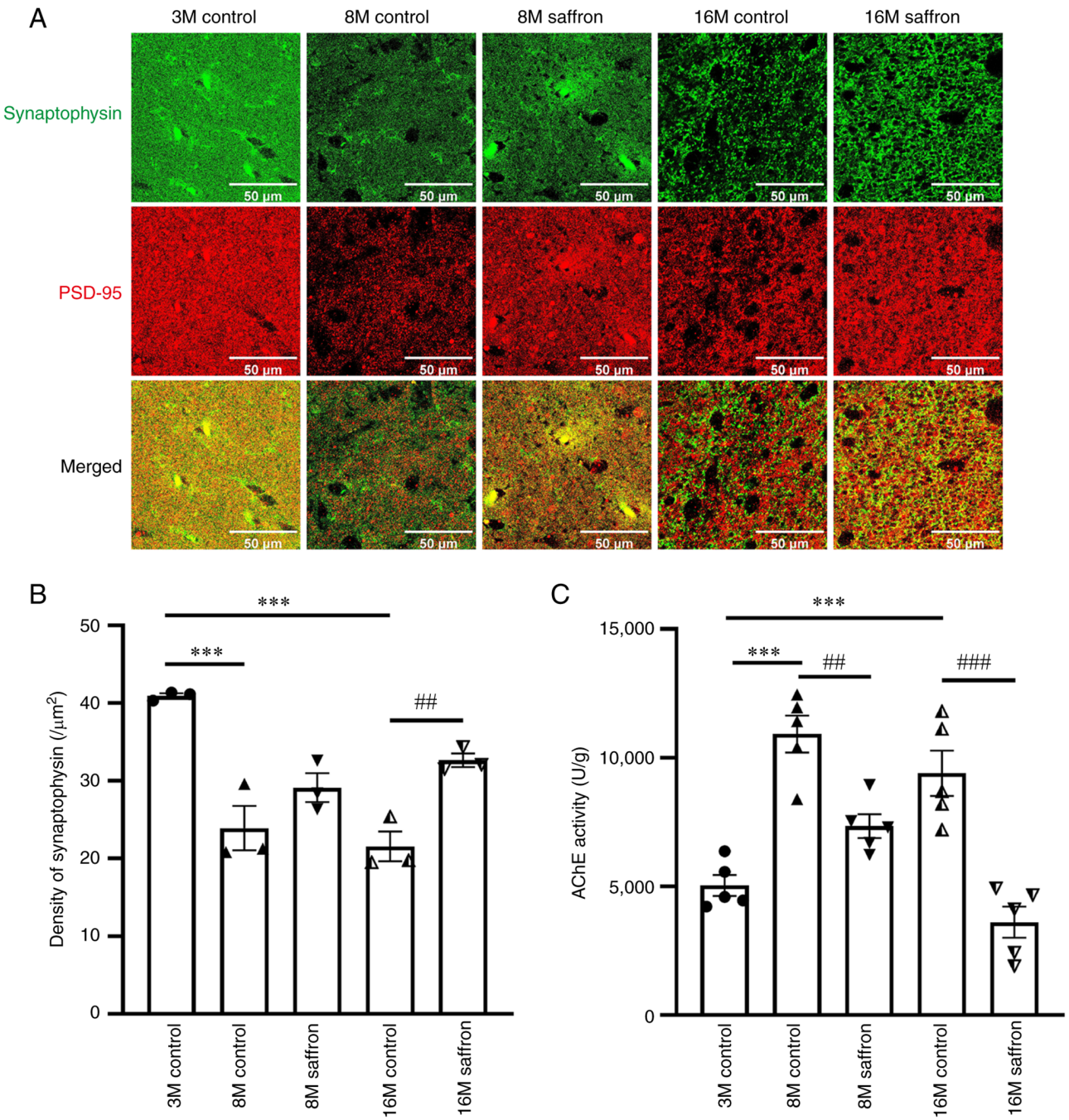

Figure 4. Effects of saffron on synaptic density and AChE activity in mice. (A) Representative immunofluorescence images of synaptophysin and PSD-95 staining in the brain (scale bar, 50 μm). (B) Synapse density and (C) AChE activity. Values are presented as the mean ± SEM. ***P<0.001 vs. the 3-month-old control group; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. the age-matched control group. AChE, acetylcholinesterase; PSD-95, postsynaptic density protein; M, month.

16-month-old group when compared with the same-aged control mice (P<0.01; Fig. 4A and B). In addition, the AChE activity in the 8- and 16-month-old control groups increased by 116.76 and 86.60% compared with that in the 3-month-old control group (both P<0.001), and decreased with saffron treatment in the 8- and 16-month-old mice (P<0.01 and P<0.001, respectively; Fig. 4C).

Effects of saffron on NAMPT and NAD⁺ content in mice. The relative expression of *Nampt* mRNA in the brain was decreased by 34.16 and 28.02% in the 8- and 16-month-old control groups compared with the 3-month-old group (both P<0.05; Fig. 5A). Following saffron treatment, *Nampt* mRNA expression significantly increased in the 8- and 16-month-old mice compared with the same-aged control mice (both P<0.01; Fig. 5A). Similar results were found regarding NAMPT content in the brain by ELISA (Fig. 5D). No significant difference in NAMPT protein levels in the brain was detected among all groups by western blotting (Fig. 5B and C). However, in the serum, NAMPT protein increased by 948.15 and 877.78% in the 8- and 16-month-old control groups compared with the 3-month-old group (both P<0.001), and these increases were attenuated by saffron treatment in the 8- and 16-month-old mice (P<0.001 and P<0.01, respectively; Fig. 5E).

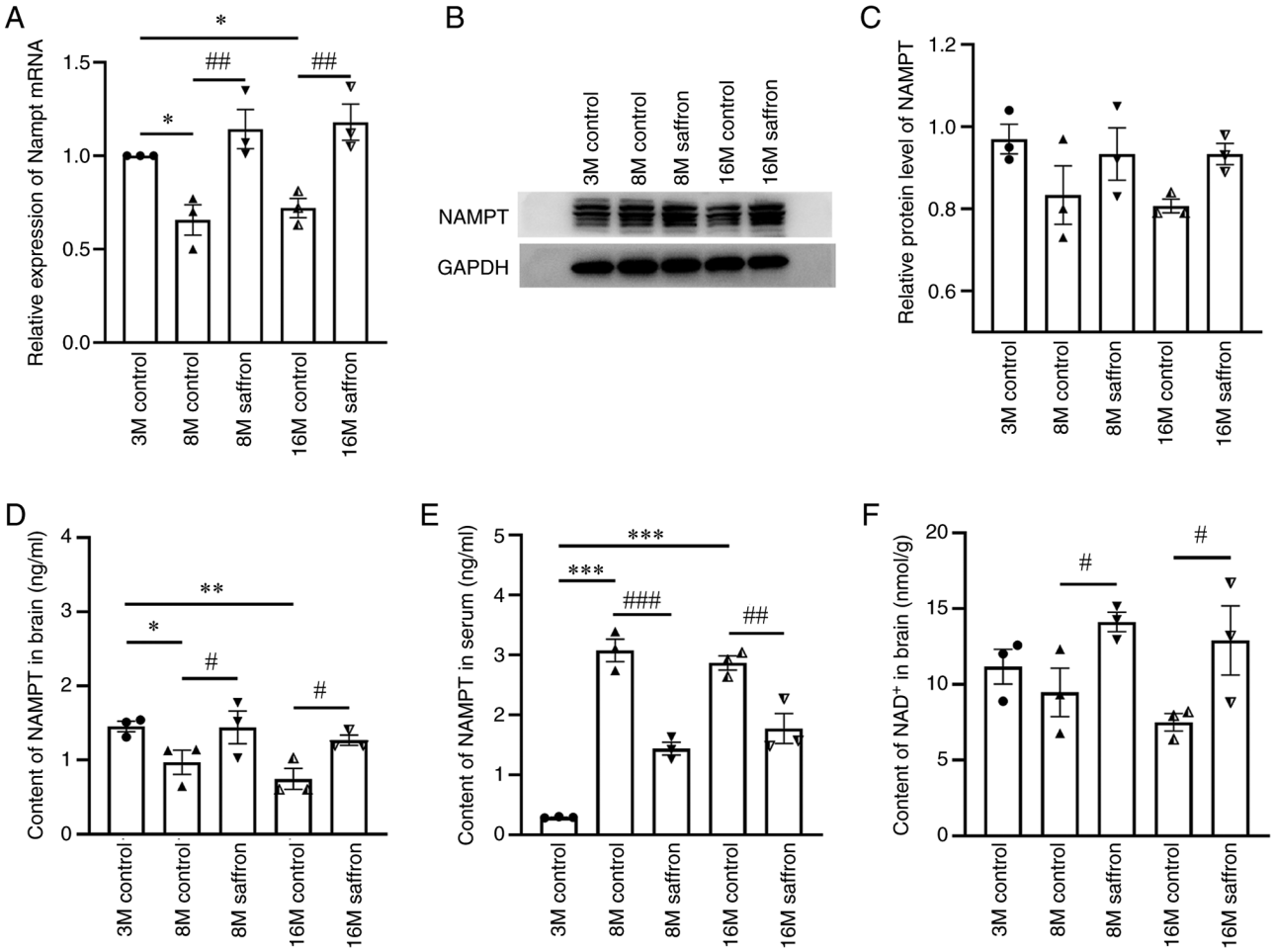

Figure 5. Effects of saffron on NAMPT and NAD+ in mice. (A) Relative expression of *Nampt* mRNA in the brain. (B) Representative western blotting bands and (C) the relative protein expression level of NAMPT in the brain. (D) Content of NAMPT in the brain and (E) serum as determined by ELISA. (F) Content of NAD+ in the brain. Values are presented as the mean ± SEM (n=3). *P<0.05, **P<0.01 and ***P<0.001 vs. the 3-month-old control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. the age-matched control group. NAMPT, nicotinamide phosphoribosyltransferase; NAD+, nicotinamide adenine dinucleotide; M, month.

The contents of NAD+ in the brain decreased with aging, albeit not significantly, and were significantly increased by saffron treatment in the 8- and 16-month-old mice (both P<0.05; Fig. 5F).

Effects of saffron on the cellular distribution of NAMPT expression. With aging, the astrocyte cell bodies were observed to increase in size and become rounder, and the protrusions from the cell body shrank. In addition, the microglia increased in size and branching. Regarding the co-distribution of with NAMPT with other proteins, the number of cells in which NAMPT was co-expressed with GFAP (both P<0.001) was significantly increased in the 8- and 16-month-old control groups compared with the 3-month-old group, as was co-expression with Iba-1 (P<0.01 and P<0.001, respectively). Further, the co-distribution of GFAP or Iba-1 with NAMPT decreased in the 8- and 16-month-old saffron groups compared with the age-matched control groups (GFAP, both P<0.01; Iba-1, P<0.05 and P<0.01, respectively; Fig. 6A, B, D and E).

With aging, neural stem cells gradually become senescent. The number of mature nerve cells decreases, activity decreases, and the renewal and replenishment ability of neurons decreases markedly (23). The co-distribution of NeuN with NAMPT in the 8- and the 16-month-old control groups decreased by 39.66 and 52.41% compared with that in the 3-month-old control group (P<0.01 and P<0.001, respectively). After saffron treatment, a significant increase in NeuN and NAMPT co-distribution was observed in the 16 month-old mice compared with the age-matched control (P<0.05); however, the increase in the 8 month-old mice was not statistically significant (Fig. 6C and F).

Effects of saffron on inflammatory reaction and oxidative stress factors in mice. The contents of TNF-α in the brains of the 8- and 16-month old control mice increased by 17.70 and 28.64% compared with those in the 3-month-old control group (both P<0.001), and the IL-1β contents were also significantly increased in the 8- and 16-month-old control groups (both P<0.01). After saffron treatment, significant reductions in TNF-α and IL-1β were observed in the 8- and 16-month-old groups compared with the corresponding age-matched controls (TNF-α, both P<0.001; IL-1β, P<0.05 and P<0.01, respectively; Fig. 7A and B). Regarding anti-inflammatory factors, significant reductions with aging in the 8- and 16-month-old control groups compared with the 3-month-old group were observed in the contents of IL-4 (both P<0.05) and IL-10 (both P<0.01) (Fig. 7C and D). The reductions in

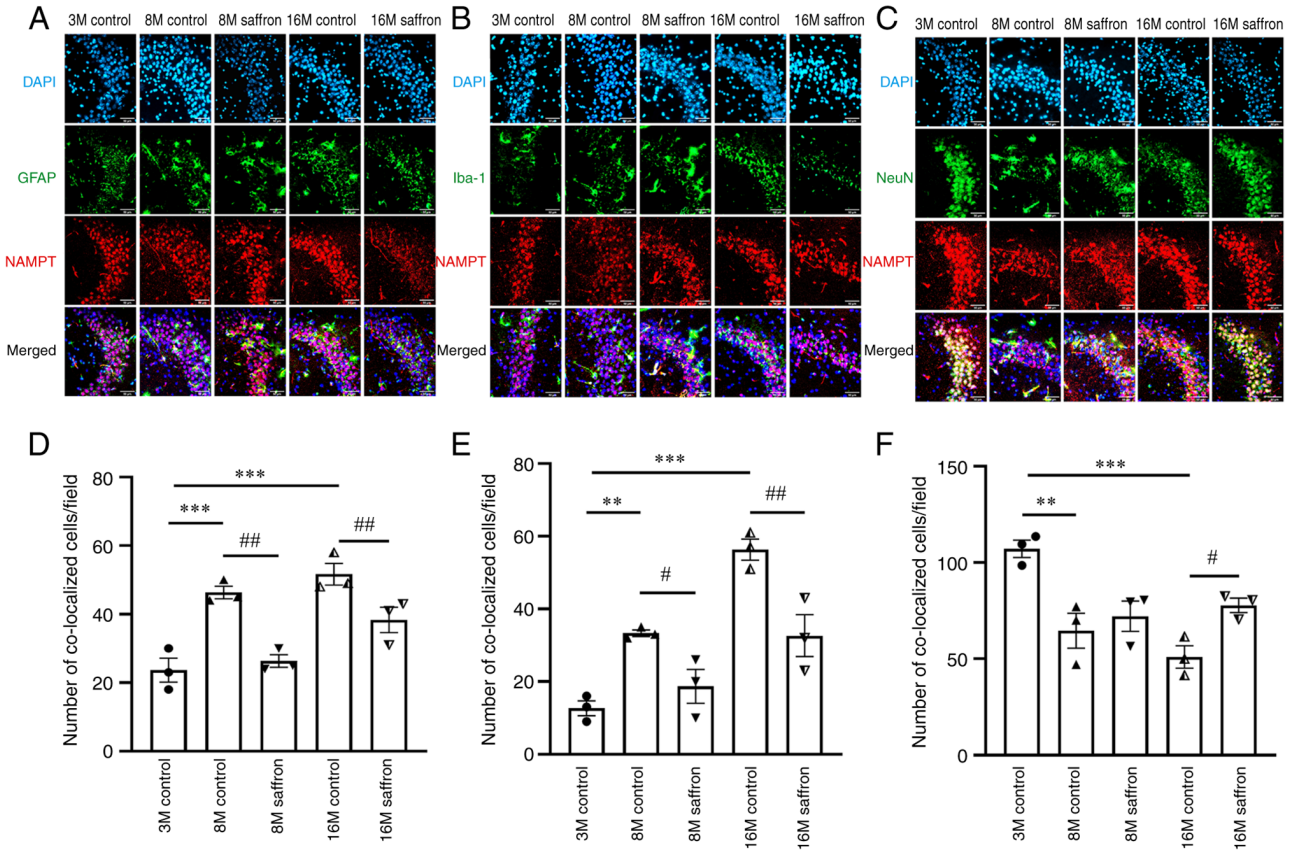

Figure 6. Effects of saffron on the cellular distribution of NAMPT. Representative images of (A) GFAP, (B) Iba-1 or (C) NeuN immunofluorescence staining with NAMPT in the hippocampus (scale bar, 50 μm). Number of cells in which (D) GFAP, (E) Iba-1 and (F) NeuN is co-distributed with NAMPT. Values are presented as the mean ± SEM. **P<0.01 and ***P<0.001 vs. the 3-month-old control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. the age-matched control group. NAMPT, nicotinamide phosphoribosyltransferase; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium-binding adapter molecule 1; NeuN, neuronal nuclei; M, month.

IL-4 content were reversed by saffron treatment in the 8- and 16-month-old saffron groups (P<0.05 and P<0.01, respectively; Fig. 7C). However, the content of IL-10 increased significantly only in the 8-month-old saffron group (P<0.01), and not in the 16-month-old saffron group (Fig. 7D).

The content of MDA increased significantly only in the 8-month-old control group compared with the 3-month-old group (P<0.05), and was decreased significantly only in the 8-month-old saffron group compared with the age-matched control (P<0.05; Fig. 7E). However, the activity of SOD in the 8- and 16-month-old control groups was significantly decreased compared with the 3-month control (both P<0.001; Fig. 7F), and the reduction was attenuated significantly with saffron treatment in the 8- and 16-month-old mice (both P<0.01; Fig. 7F).

Discussion

In the present study, the senescence-delaying effects of saffron in mice were investigated. These manifested as improvements in cognition dysfunction, the pathological morphology of the tissues, and increased synaptic density and decreased AChE activity in the brain. We hypothesize that these changes may be associated with changes in the content and cellular distribution of NAMPT, followed by an increase in NAD^+ production.

In order to evaluate whether saffron met the requirements of the Chinese Pharmacopoeia, which comprise a total crocin content of >10%, the contents of picrocrocin and crocin were detected by HPLC prior to use of the saffron extract. The results indicated that the quality of saffron used in the present study was suitable for investigating the senescence-delaying effects of saffron in aging mice. The quality of the saffron was similar to that in our previous study and another phytochemical study (7,24). With consideration of the pharmacokinetic properties of saffron (25), it is proposed that it may be more valuable to study the effects of saffron extract than those of its active components for use in healthcare. However, in order to clarify the mechanism of the senescence-delaying effects of saffron extract, it will be essential to use molecular docking techniques to screen its active components and conduct further experiments to verify their effects.

Several prior studies have reported that the morphology of the liver (26), kidneys (27) and skin (28) all change with aging, including the development of pycnotic nuclei in the liver, thinner dermal collagen fibers, lower infiltration of inflammatory cytokines in the skin, and less hemorrhaging and ballooning in the kidneys. In the present study, the senescence-delaying effects of saffron were also indicated by amelioration of the pathophysiological changes in the liver, skin and kidneys.

Behavioral tests, including the OFT and BMT, are often applied to measure the spontaneous activity and learning-memory function of mice, which decrease in an

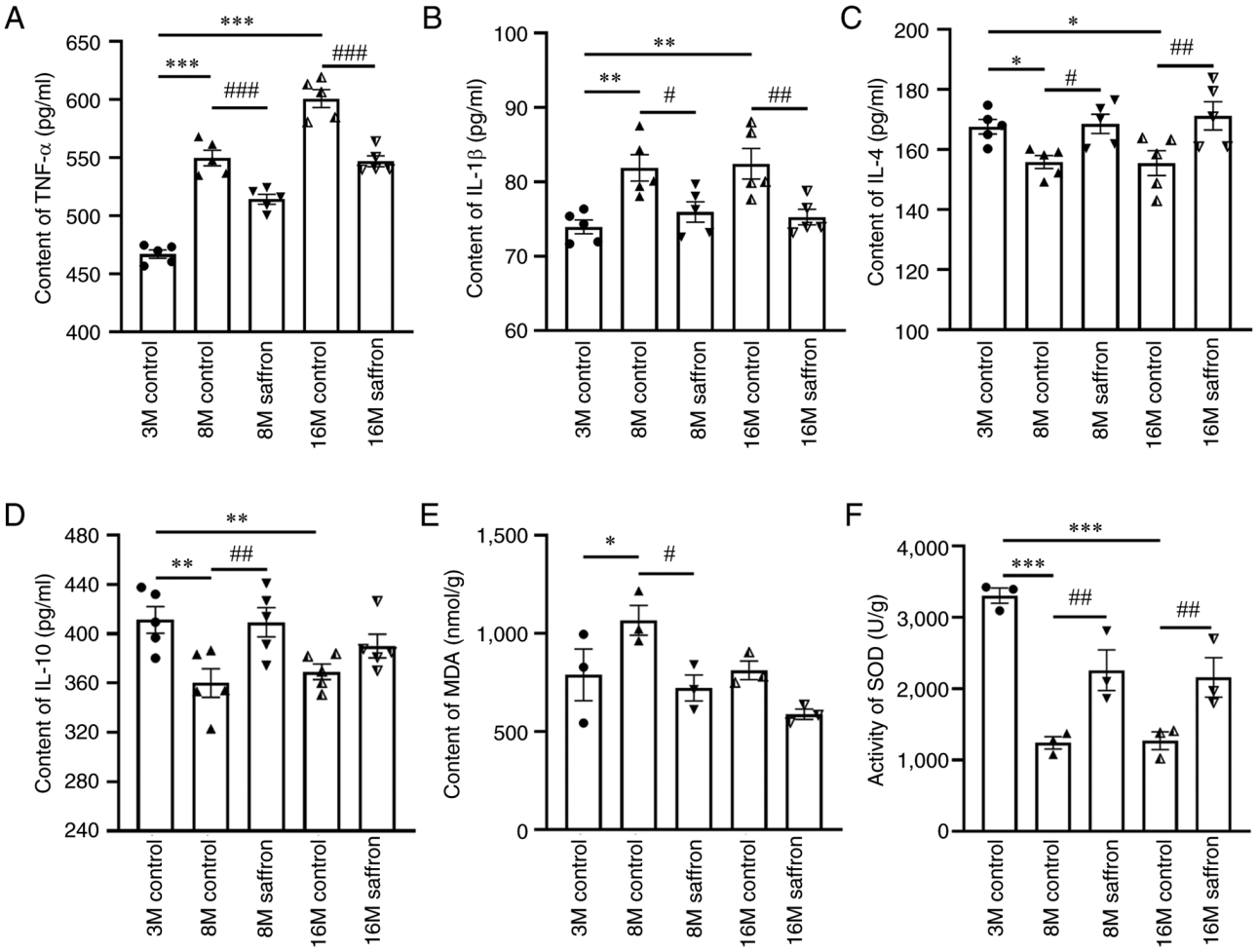

Figure 7. Effects of saffron on inflammatory and oxidative stress factors in mice. Contents of (A) TNF-α, (B) IL-1β, (C) IL-4, (D) IL-10 and (E) MDA, and (F) SOD activity in the brain. Values are presented as the mean ± SEM. *P<0.05, **P<0.01 and ***P<0.001 vs. the 3-month-old control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. the age-matched control group. TNF, tumor necrosis factor; IL, interleukin; MDA, malondialdehyde; SOD, superoxide dismutase; M, month.

age-dependent manner (29). In the present study, the results of these tests showed decreased locomotor activity in the 8- and 16-month-old control mice and increased probe safety hole latency in 16-month-old mice, which are key signs of aging (30). An increasing tendency in the number of explorations of the target hole was observed after saffron treatment. We hypothesize that the insignificance of the behavioral improvements achieved by saffron could be associated with the ages of the animals. Shoji and Miyakawa (31) previously reported that 46- to 72-week-old mice were middle-aged and 98-week-old mice were aged. However, in the present study 8- and 16-month-old mice were considered as middle-aged and aged, respectively. The senescence-delaying effects of saffron may be more pronounced in older mice, and BMT or other methods should be used in further studies to investigate this.

Certain indicators associated with learning-memory function change with aging, such as synaptic density, AChE activity, and performance in Morris water maze and novel object recognition tests (32). Several reports have suggested that the immunoreactivity of synaptophysin reduces with aging, which would cause learning and memory dysfunction (33,34). Consistent with these previous findings, the present study found that synaptophysin density decreased with aging, while saffron increased synaptophysin density in 16-month-old mice. AChE decomposes acetylcholine (ACh) and inhibits ACh-induced signal transmission; ACh is an important neurotransmitter that is released by cholinergic neuron terminals, participates in the transmission of neural signals and is closely associated with learning and memory function (35). The present study found that AChE activity increased in the 8- and 16-month-old mice, and saffron reduced its activity in mice of both ages. Furthermore, the reduction was greater in the 16-month-old mice. These results suggest that saffron can improve indicators of learning and memory impairment associated with aging, but this it has not been significantly reflected in the BMT results, and requires further verification using other tests.

NAMPT is a rate-limiting enzyme in mammalian NAD^+ biosynthesis and the controller of intracellular NAD^+ levels (13). NAMPT-mediated NAD^+ biosynthesis plays an important role in energy metabolism, DNA repair, chromatin remodeling, cell aging and immune cell function regulation (36). The level of NAMPT decreases significantly with aging, which leads to the development of age-related diseases including metabolic diseases, neurodegenerative diseases and cancer (37). NAMPT exists in two forms in mammals, namely intracellular NAMPT (iNAMPT) and extracellular NAMPT (eNAMPT). iNAMPT is predominantly found in the brain, while eNAMPT mainly exists in the serum. iNAMPT

protects cells predominantly by promoting the synthesis of nicotinamide mononucleotide, a precursor of NAD (17,38,39). Conversely, eNAMPT can function either as an enzyme for NAD biosynthesis (40), or a proinflammatory cytokine (17,41).

It has previously been shown that level of eNAMPT in the serum increases and that of iNAMPT in the brain decreases upon aging (15); the latter is consistent with the ELISA findings for NAMPT in the present study. The reduction of eNAMPT achieved following saffron treatment indicates alleviation of the inflammation that occurs upon aging. Although that mRNA level of *nampt* was shown to decrease in the brain with aging, no difference in the protein expression of NAMPT was observed among the groups. We hypothesize it could be associated with a change in the cellular distribution of iNAMPT, resulting in its secretion out of the brain. Ma *et al* (42) previously reported similar findings regarding *Nampt* mRNA and NAMPT protein expression.

The cellular distribution of iNAMPT changes with aging, which manifests as an increase in NAMPT expression in the microglia and astrocytes, and a reduction in NAMPT expression in neurons (15,43). Microglia and astrocytes are inflammatory cells of the central nervous system that possesses a heightened reactivity in the aged brain (44), and iNAMPT has been shown to play a role in regulating their inflammatory reaction (45). Furthermore, it has been reported that oxidative stress (46) and inflammatory reactions (47,48) are both associated with aging. As such, the increase in NAMPT in neurons and reduction in the microglia and astrocytes may be a mechanism underlying the alleviation of inflammatory and oxidative stress reactions after saffron treatment in middle-aged and aged mice (49,50).

Although senescence-delaying effects and changes of NAMPT-NAD^+ contents and cellular distribution induced by saffron were found in the present study, the association between these effects is unclear. However, according to the literature (15,43) and findings on aging from the present study, it is speculated that NAMPT-NAD^+ may have a certain relationship with the senescence-delaying effects of saffron. However, the exact association between them requires further study. In the future, *Nampt*$^{flox/flox}$ mice (51) will be used to explore and clarify the mechanism by which saffron extract and its active components affect the NAMPT-NAD^+ system and induce senescence-delaying effects.

In summary, the present study showed that saffron treatment changed the content and cellular distribution of NAMPT in the brain and serum induced by aging in mice. These changes could be partly associated with improvements in physiological state of aging, learning-memory dysfunction, and microglia- and astrocyte-mediated inflammatory reaction. Therefore, the findings of the study suggest that saffron has potential as a treatment to delay senescence.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

LXiao contributed to the animal model, behavioral tests, data collection and analysis, as well as writing the original draft of the manuscript, writing-review and editing. RS contributed to the animal model, data collection and analysis. YH and LXia contributed to the animal model and behavioral tests. KL helped with behavioral testing and funding acquisition. WF also helped with behavioral testing while KZ contributed to the animal model and data collection. YY was responsible for project administration and design, data collection and analysis, writing-review and editing and funding acquisition. LXiao and YY confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The animal experiment was approved (approval no. 2021-040) by the Animal Health, Ethics and Research Committee of Hangzhou Medical College (Hangzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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