Effects of aged garlic extract on aging-related changes in gastrointestinal function and enteric nervous system cells

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Abstract. Dysmotility of the gastrointestinal (GI) tract is commonly seen in elderly individuals, where it causes significant morbidity and can lead to more severe conditions, including sarcopenia and frailty. Although the precise mechanisms underlying aging-related GI dysmotility are not fully understood, neuronal loss or degeneration in the enteric nervous system (ENS) may be involved. Aged garlic extract (AGE) has been shown to have several beneficial effects in the GI tract; however, it is not known whether AGE can improve GI motility in older animals. The aim of the present study was to examine the effects of AGE on the ENS and gut motility in older mice and elucidate potential mechanisms of action. An AGE-formulated diet was given to 18-month-old female mice for 2 weeks. Organ bath studies and cell culture demonstrated that AGE: i) Altered gut contractile activity; ii) enhanced viability of ENS cells; and iii) exhibited neuroprotective effects on the ENS via reduction in oxidative stress. These findings suggest that AGE could be used to develop novel dietary therapeutics for aging-related GI dysmotility by targeting the associated loss and damage of the ENS.

Introduction

Aging is a major risk factor for various diseases and disorders (1) and leads to a decline in the biological functions of multiple organs, including the gastrointestinal (GI) tract (2). Aging-related GI disorders, including esophageal reflux, dysphagia, chronic constipation, and fecal incontinence (3), can also be associated with sarcopenia and frailty leading to increased overall mortality (4,5). Approximately half of people 65 years or older experience at least one significant GI symptom (6). Dysphagia was found in approximately one third of community-dwelling elderly, and more than half of elderly nursing home residents (7). Almost one-third of adults 60 years or older report at least occasional constipation (8). Also, GI disorders are more prevalent in women than men, and the prevalence of several GI disorders increases with age (9,10). GI disorders are more common in females due to various factors including reproductive hormones (11,12), gut sensation (10), and mental disorders (13-15). Despite high prevalence, the treatment options for aging-related GI disorders are limited, therefore novel therapeutic approaches are warranted to improve the quality of life of elderly individuals. The enteric nervous system (ENS) is an extensive network of neurons and glial cells within the wall of the GI tract and plays a critical role in regulating gut motility and other fundamental gut functions (16,17). ENS abnormalities and GI dysfunction including neuronal loss and slow transit, respectively have been well described in older animals and humans and are believed to be one of the etiologies of aging-related GI disorders (18-21). Therefore, targeting the ENS may be a novel therapeutic approach for these conditions (19).

Aged garlic extract (AGE) is a unique garlic product produced by aging garlic in a water-ethanol process for more than 10 months. During this time, sulfur-containing amino acids including S-allylcysteine (SAC), S-1-propenylcysteine (S1PC), and S-allylmercaptocysteine (SAMC) are produced (22,23). Several clinical trials have shown that AGE has beneficial effects in patients with hypertension (24-27) and gingivitis (28,29). Increasing evidence indicates that SAC and S1PC possess diverse medical benefits, including anti-hypertensive (30-34), anti-oxidant (35-37), anti-aging (38,39), and anti-inflammatory (40-44) properties. Several GI studies have shown that AGE changes microbiota composition (27,45,46), attenuates inflammation in an animal model of colitis (44), and protects epithelial cells from methotrexate-induced cytotoxicity (47). However, the effects of AGE on GI motility and ENS in older subjects are not well understood. In this study we investigated the effects of AGE on gut motility and ENS cells using old mice and explored potential mechanisms of action.

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Materials and methods

Animals. All animal protocols were approved by the Institutional Animal Care and Use Committee at Massachusetts General Hospital (Protocols #2009N000239 an #2013N000115). All methods were performed in accordance with relevant regulations. Females of C57BL/6J (Stock #007914) mice, both 2-3-month-old (Young) and 18-month-old (Old), were purchased from Jackson Laboratory (Bar Harbor, ME). 18-month-old mice represent approximately 56 years of age in humans (48,49). For generation of dual reporter mice in which enteric neurons express tdTomato and enteric glia/progenitors express GFP, *Plp1*^{GFP} mice (50) were kindly gifted by Dr. Wendy Macklin, University of Colorado, Denver. To obtain *Plp1*^{GFP}*;Actl6b::Cre; ROSA26-tdTomato* (annotated as Plp1^{GFP}; Baf53b-tdT) mice, *Actl6b::Cre* mice (Stock #027826) were crossed with *Plp1*^{GFP} mice, and their offspring were crossed with *ROSA26-tdTomato* mice (Stock #007914) (51). In order to isolate longitudinal muscle layer with myenteric plexus (LMMP) and enteric neural cells animals were euthanized by carbon dioxide overdose, which is displacement of chamber air with compressed carbon dioxide at 30-70% per min. Death of animals was confirmed by lack of chest movement and heartbeat, after which cervical dislocation was performed. Both male and female mice were used for *the in vitro* studies as the influence of factors such as gut sensation and reproductive hormons can be excluded.

Chemicals. AGE was prepared from cloves of garlic (*Allium sativum* L.) through a process of rinsing with purified water, slicing, soaking in ethanol 20-50% (v/v), and extracting/aging for more than 10 months (52). AGE powder was obtained from Wakunaga Pharmaceutical Co. ltd and sent to Bio-Serv (Flemington, NJ) to prepare 3% AGE-formulated diet using standard mouse chow (AIN-93G, Bio-Serv, NJ). The AIN-93G the purified rodent diet that containing 200 g Casein, 100 g Sucrose, 397.5 g Cornstarch, 132 g Dyetrose, 3 g L-cystine, 50 g Cellulose, 70 g Soybean oil, 0.014 g t-Butylhydroquinone, 35 g Mineral mix, 10 g Vitamin mix, and 2.5 g Choline bitartrate per kg of diet (53). The 3% AGE-formulated diet was given to female mice for 2 weeks after the mice were randomized based on body weight. Two weeks after the initiation of feeding the AGE-formulated diet, *in vivo* assays and analysis of oxidative stress in myenteric plexus were performed. For *in vitro* and *ex vivo* studies, AGE powder was dissolved in sterile PBS and the solution was passed through with a 0.22 μM filter.

Gastrointestinal transit time. Total gastrointestinal transit time was measured as previously described (54). Mice (n=6, each group) were acclimatized for 30 min individually in cages without bedding, and 0.15 ml of 6% (w/v) carmine red dye (Sigma, C1022) in 0.5% (w/v) methylcellulose was administered to each mouse by oral gavage. The time from gavage to the appearance of the first red pellet was recorded as total gastrointestinal transit time. Maximum observation time was 6 h.

Bead expulsion test. Mice (n=6, each group) were fasted overnight before the test was performed as described previously (55). Mice were acclimatized for 30 min, then a 3-mm glass bead (Sigma, #1040150500) was inserted into the rectum of each mouse using a silicone pusher under anesthesia by isoflurane (Covetrus, #11695-6777-2). Isoflurane, at 3 and 2%, was used for induction and maintenance, respectively. After bead insertion, mice were placed in individual plastic cages. The time to evacuate the bead started after the mice recovered from the anesthesia.

Fecal pellet output. Fecal pellet output was measured as reported previously (56) in individual metabolic cages for 24 h (n=6, each group). The weight of food consumed over 24 h was obtained. Pellet number was calculated using the average weights of dry feces (n=25) per mouse.

Fecal water content. The previously described method was used (57). Each mouse (n=6, each group) was placed in a plastic cage individually and wet fecal pellets were collected for 2 h. The wet fecal pellets were dried in the oven at 60˚C for 48 h. Fecal water content was calculated according to the following equation: (wet fecal weight-dry fecal weight)/wet fecal weight x100.

Organ bath measurements of colonic smooth muscle activity. The organ bath experiments with colon rings followed well-established protocols. We conducted pilot studies to optimize parameters such as intensity (40-50 volts), frequency (5 Hz), and pulse duration (0.3 ms) to ensure reproducible and physiologically relevant responses for assessing smooth muscle contractility. These parameters have been validated in our laboratory, and experiments were performed using sparameters previously described (58-61). Freshly excised segments of distal colon were immediately placed in oxygenated Krebs solution (118 mmol/l NaCl, 4.7 mmol/l KCl, 1.2 mmol/l $MgSO_4{\cdot}7H_2O$, 1.2 mmol/l KH_2PO_4, 25 mmol/l $NaHCO_3$, 11.7 mmol/l glucose, and 1.25 mmol/l $CaCl_2$) at 37˚C. Tissue rings, approximately 5 mm in length, were mounted between two metal hooks attached to force displacement transducers in a muscle strip myograph bath (Model 820 MS; Danish Myo Technology, Aarhus, Denmark) containing 7 ml of oxygenated Krebs solution. The rings were gently stretched to establish a basal tension of 0.5 g and allowed to equilibrate for 30-45 min, with Krebs solution being replaced every 20 min. Spontaneous contractions were recorded in both the absence and presence of AGE (1% w/v). Afterwards, contractions were recorded again following the addition of the nitric oxide synthase inhibitor L-NAME (100 mM; Sigma-Aldrich, St. Louis, MO). Electrical field stimulation (EFS) was then applied to the tissue using a pulse train of 40-50 V (15-sec duration, 300 μs pulse width, 5 Hz frequency) via a CS4+ constant voltage stimulator controlled by MyoPulse software (Danish Myo Technology). The procedure was repeated after the addition of AGE (1% w/v). Force contraction data from the circular smooth muscle were recorded and analyzed using a Power Lab 16/35 data acquisition system and LabChart Pro Software v8.1.16 (ADInstruments, NSW, Australia). Tissue viability was confirmed by assessing the contraction response to 60 mM KCl at the conclusion of the experiment. Baseline spontaneous activity was quantified by measuring the area under the curve (AUC), from 60 sec of data collected 5 min

before the addition of AGE or L-NAME This was compared to the response following AGE or L-NAME, which was similarly quantified by measuring the AUC, from 60 sec of data collected immediately after the addition of AGE or L-NAME. Baseline maximum values were determined by averaging 60 sec of data recorded 1 min before EFS application. Changes in contraction were measured from the first 60 sec after stimulation onset and expressed as absolute differences from baseline. EFS was applied three times at 5-min intervals, and the maximum response was calculated as the mean of the three trials.

Isolation and expansion of mouse enteric neural cells. Enteric neural cells, including enteric neural stem cells (ENSCs), were isolated from mice as previously reported (62-64). Briefly, LMMP was separated from large intestine of mice (young, old, and $Plp1^{GFP}$; Baf53b-tdT). Enzymatic dissociation was achieved using dispase (250 μg/ml, STEMCELL Technologies, Vancouver, Canada) and collagenase XI (1 mg/ml, Sigma Aldrich, St. Louis, Missouri) at 37˚C for 45 min. Single cells were isolated by filtration through a 40-μm filter and plated at $5x10^5$ cells/ml in a 25-cm^2 flask in a 1:1 mixture of DMEM (Thermo Fisher Scientific) and NeuroCult Mouse Basal Medium (StemCell Technologies) supplemented with 1% penicillin/streptomycin (Gibco, #15140122), 20 ng/ml insulin growth factor (StemCell Technologies), and 20 ng/ml basic fibroblast growth factor (StemCell Technologies), 2% B27 supplement (gibco), 1% N2 supplement (gibco), 50 μM beta-mercaptoethanol (gibco), and 75 ng/ml retinoic acid (Sigma Aldrich). After 7 days in culture, primary neurospheres were obtained.

Neurosphere assay and cell viability assay. Primary neurospheres from wildtype C57BL/6J (n=2, male and female) and $PLP1^{GFP}$; BAF53b-tdT mice (n=2, male and female) were dissociated by Accutase (StemCell technologies, # 7920). 5,000 cells/well were plated into 96-well plate (CORNING, #3474) and secondary neurospheres treated with AGE at 0.25 to 1 mg/ml in culture media for 7 days (n=3, each group). The samples were dissociated with dispase and collagenase XI to generate single cell suspension and fixed with 4% PFA for 15 min. Random images of secondary neurospheres from wildtype C57BL/6J were taken using a Keyence BZX-700 All-In-One Microscope (Keyence America Itasca, IL) and the number of neurospheres from wildtype C57BL/6J quantified by ImageJ software (NIH). A cell viability assay was performed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA, PAG7570). Secondary neurospheres from $Plp1^{GFP}$; Baf53b-tdT mice were dissociated to single cells and tdT+ and GFP+ positive cell numbers were counted using ImageJ software (NIH).

EdU proliferation assay and immunohistochemistry. Primary neurospheres from wildtype C57BL/6J mice (n=2, male and female) were generated as described previously. AGE (1 mg/ml) was added to the media for secondary neurospheres in the presence of 10 μM of 5-ethynyl-2'-deoxyuridine (EdU) for 2 days (n=3, each group). After removing the media, secondary neurospheres were incubated in new AGE-containing media for 5 days. The samples were dissociated with dispase and collagenase XI to generate single cell suspension fixed with 4% PFA for 15 min and Click-iT EdU Cell Proliferation Kit for Imaging (Fisher Scientific, C10340) was performed. For immunohistochemical staining, 10% donkey serum and 1% Triton X-100 in phosphate-buffered saline (PBS) was used for blocking. Primary antibodies, including human anti-HuC/D (Anna1, 1:20, kindly gifted by Lennon lab) and rabbit anti-P75 (1;400, Promega, G3231), were incubated overnight at 4˚C, followed by secondary antibodies for 1 h. Secondary antibodies included anti-human IgG (1:200, Alexa Fluor 594, Jackson ImmunoResearch) and anti-rabbit IgG (1:200; Alexa Fluor 488, Invitrogen). Random Images were taken from each group using a Keyence BZX-700 All-In-One Microscope (Keyence America Itasca, IL) and the number of double-labelled Hu+EdU+ or P75+EdU+ cells counted using ImageJ software (NIH).

Oxidative stress in myenteric plexus. MitoSOX (Thermo Fisher Scientific, M36008) was used to identify mitochondrial-derived production of superoxide in the myenteric ganglia of the ENS (65). Fresh colonic LMMP preparations were collected from the mice (n=4, each group). The samples were incubated in Hanks' Balanced Salt Solution (HBSS, Thermo Fisher Scientific) containing 5 μM of MitoSOX at 37˚C for 30 min. Tissues were washed with PBS and fixed with 4% PFA overnight at 4˚C. Images were captured, converted into binary format, and area of fluorescence within ganglia was measured in arbitrary units using ImageJ software (NIH).

ENS cell culture and evaluation of the effects of AGE. LMMPs were separated from colons dissected from 2 months old $Plp1^{GFP}$; Baf53b-tdT mice (n=2, male and female) and dissociated enzymatically using dispase (250 μg/ml, STEMCELL Technologies) and collagenase XI (1 mg/ml, Sigma-Aldrich) at 37˚C for 45 min. Counter filtration was performed using a 20-μm cell strainer (pluriSelect, #43-50020-01) as previously (66). Samples were centrifuged at 350 G for 5 min and resuspended in NeuroCult Mouse Basal Medium (StemCell Technologies) containing 10% FBS and 1% penicillin/streptomycin (Gibco, #15140122). Isolated enteric ganglia in the media were plated into fibronectin (Sigma-Aldrich, #F1141)-coated 48 well plates and cultured for 24 h. Pre-treatment of AGE without hydrogen peroxide (H_2O_2) was performed for 48 h. Then, PBS treatment as control, 100 μM of H_2O_2 (Sigma-Aldrich, H1009) alone, and co-treatment of AGE and 100 μM of H_2O_2 were carried out for 24 h (n=3, each group). The samples were fixed with 4% PFA for 15 min at RT. Random images were taken using a Keyence BZX-700 All-In-One Microscope (Keyence America Itasca, IL). The number of neurons and glial cells, and neurite length, were quantified using ImageJ software (NIH).

Statistical analysis. Data analysis was performed using GraphPad Prism v10 (GraphPad Software, Inc., San Diego, CA). Two-tailed t-tests were performed for pairwise comparisons. A one-way analysis of variance (ANOVA) was performed with a post hoc Dunnett's test and Tukey's test for multiple comparisons. For all analyses, $P<0.05$ was considered significant. All data are presented as mean ± SEM, unless otherwise stated.

Results

AGE improves colorectal dysfunction in old mice. To characterize gastrointestinal (GI) motility in old mice, we performed multiple *in vivo* functional assays in young (2-3-month-old) and old (18-month-old) mice (Fig. 1A). Both total GI transit time (Fig. 1B, 2.98±0.2 h in young vs. 5.01±0.1 h in old, P<0.0001) and rectal bead expulsion time (Fig. 1C, 2.78±0.2 min in young vs. 6.47±0.3 min in old, P<0.0001) were significantly delayed in old mice. Furthermore, significantly decreased fecal pellet output (Fig. 1D, 127±7.8 in young vs. 35.7±8.9 in old, P<0.0001) and fecal water content (Fig. 1E, 42.7±2.2% in young vs. 33.2±1.9% in old, P<0.01) were observed in old mice despite no significant difference in food intake (Fig. 1F, 3.10±0.2 g in young vs. 2.37±0.3 g in old, ns).

AGE-formulated diet was given to old mice for 2 weeks and the same GI functional analyses were performed (Fig. 1A). Interestingly, all GI functional parameters, including total GI transit time (Fig. 1B, 5.01±0.1 h in old vs. 4.29±0.2 h in old+AGE, P<0.05), rectal bead expulsion time (Fig. 1C, 6.47±0.3 min in old vs. 3.87±0.4 min in old+AGE, P<0.0001), fecal pellet output (Fig. 1D, 35.7±8.9 in old vs. 120±14 in old+AGE, P<0.001), and fecal water content (Fig. 1E, 33.2±1.9% in old vs. 43.2±1.0% in old+AGE, P<0.01) were significantly improved by the AGE-diet. Food intake was not significantly reduced by AGE-diet (Fig. 1F, 2.37±0.3 g in old vs. 2.87±0.4 g in old+AGE, ns). These findings suggest that AGE has the potential to restore aging-related GI dysmotility in mice.

AGE increases smooth muscle relaxation of aged colon via nNOS signaling. To evaluate the effect of AGE on colonic smooth muscle contractility, we performed organ bath studies on colon from 18-month-old mice. The representative tracings of each group during the baseline recording (Fig. 2A) and in response to electrical field stimulation (EFS) (Fig. 2B) are shown. In the presence of AGE, both baseline contractile activity (Fig. 2A) and EFS induced responses (Fig. 2B) were reduced compared to non-AGE-treated tissues. Quantitative analysis, determined by measuring area under the curve (AUC) and the amplitude of EFS responses, were significantly reduced in the presence of AGE (Fig. 2C, 16.4±1.8 g.s in absence of AGE vs. 13.4±1.7 g.s in presence of AGE, P<0.05; Fig. 2D, 2.41±0.4 g in absence of AGE vs. 1.75±0.3 g in presence of AGE, P<0.05). These changes in response to AGE were attenuated in the presence of L-NAME (N(ω)-nitro-L-arginine methyl ester), an inhibitor of neuronal nitric oxide synthase (nNOS) (Fig. 2E, 20.0±5.1 g.s in absence of AGE vs. 19.1±4.8 g.s in presence of AGE, ns), suggesting that the effect of AGE on colonic contractility is mediated, at least in part, by nNOS.

AGE leads to expansion of cultured ENS cells. Recent evidence has demonstrated active regeneration and remodeling of the ENS postnatally (67). To evaluate the effect of AGE on ENS homeostasis in old mice, we isolated enteric neuronal stem/progenitor cells (ENSCs) from the colon of young and old mice.

Interestingly, viability of ENSCs isolated from old mice was significantly reduced in comparison to young-derived ENSCs (Fig. 3A, 100±5.0% in young ENSC vs. 14.8±0.3% in old ENSC, P<0.0001), and the number of old-mouse-derived ENSCs was also significantly lower (Fig. S1A, 4.9±0.4 in young ENSC vs. 2.5±0.4 in old ENSC, P<0.05). Addition of AGE to the culture media increased cell viability in and the number of both young- and old-mouse-derived ENSCs (Figs. 3B and S1B). We isolated ENSCs from $Plp1^{GFP}$; Baf53b-tdT mice in which enteric neurons express tdTomato and enteric glia/progenitors express GFP (51), and cultured them in the presence or absence of AGE (Fig. 3D-I). Quantitative analysis demonstrated that AGE significantly increased the Plp1-GFP population in a dose dependent manner (Fig. 3F and G, yellow arrows; Fig. 3J). AGE also expanded the neuronal (Baf53b-tdT positive, Fig. 3D and E, white arrows; Fig. 3K) population, but only at the 1 mg/ml concentration (Fig. 3K, 7.41±0.8 in control vs. 12.3±0.6 in AGE 1 mg/ml, P<0.01).

AGE promotes proliferation of enteric neural cells. We tested whether AGE activates proliferation in ENS cells using the thymidine analogue, EdU (5-Ethynyl-2'-deoxyuridine). Isolated ENSCs were cultured in the absence (Control) or presence of AGE (1 mg/ml) for 2 days in medium containing EdU. We replaced the culture medium on day 3 and maintained the culture for an additional 5 days. Immunofluorescent staining was performed using anti-Hu and anti-P75 antibodies (Fig. 4A-H) to label neurons and glia/ENS progenitors, respectively. AGE promoted cell proliferation, as shown by an increase in neurons that are double positive for EdU+/Hu+ (Fig. 4I, 1.87±0.2 in control vs. 5.87±0.5 in AGE 1 mg/ml, P<0.01) and an increase in EdU+/P75+ as glia/progenitors (Fig. 4J, 1.43±0.2 in control vs. 3.41±0.2 in AGE 1 mg/ml, P<0.01).

AGE reduces oxidative stress in ENS from old mice. Neurons are susceptible to oxidative stress (68), which plays a role in aging-related enteric neuronal damage (69). Therefore, we evaluated reactive oxygen species (ROS) within the myenteric plexus of the muscular layer of mouse colon using MitoSOX labeling. The ROS visualized by MitoSOX in old mice (Fig. 5B and E) (Fig. 5E, 48.7±1.5 in young vs. 117±13 in old, P<0.001) was significantly more prominent compared to that in young mice (Fig. 5A and E), supporting the idea that aging increases oxidative stress in the ENS. Old mice fed an AGE-formulated diet for 2 weeks (Fig. 1A) demonstrated significantly reduced ROS in the myenteric plexus (Fig. 5C and E) (Fig. 5E, 117±13 in old vs. 74.7±6.1 in old+AGE, P<0.05). These findings suggest that AGE ameliorates oxidative stress in the ENS of old mice.

AGE protects against oxidative stress-induced degeneration of cultured enteric neural cells. Several recent studies have shown that prevention of oxidative stress could be a novel therapeutic strategy for neurodegenerative disorders (70-72). Here, we tested whether AGE has neuroprotective effects on cultured ENS cells. Hydrogen peroxide (H_2O_2) is commonly used to induce neuronal damage via oxidative stress (73,74). We isolated ENS cells from $Plp1^{GFP}$; Baf53b-tdT mice and cultured them in the absence (Fig. 6A-C) or presence of H_2O_2 (Fig. 6D-F). In the presence of H_2O_2, there was a significant reduction in the number of tdT+ neurons (Fig. 6M, 37.3±3.2 in control vs. 5.00±0.6 in H_2O_2, P<0.0001) and GFP+

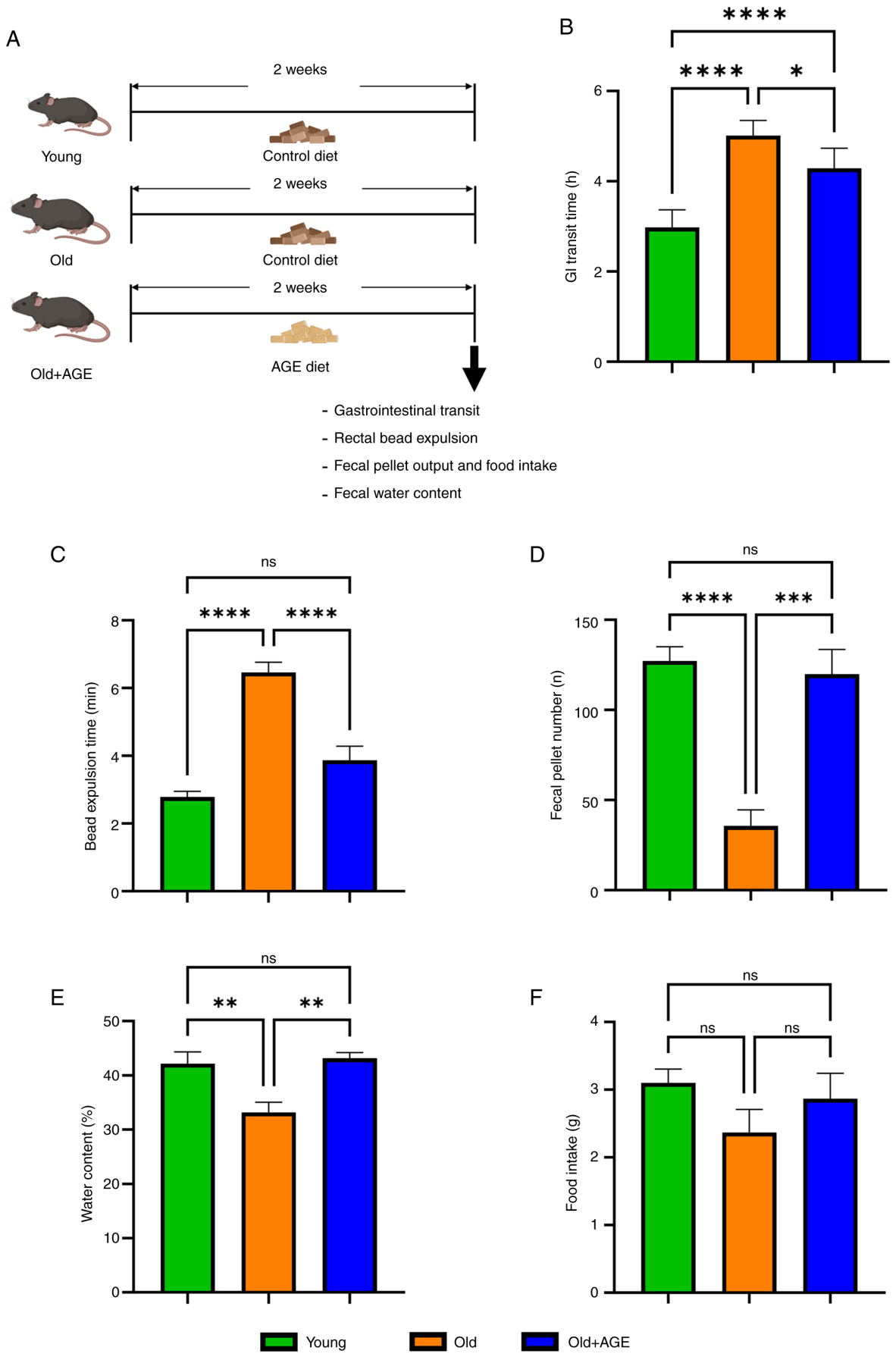

Figure 1. Effects of AGE on aging-related colorectal dysfunction in old mice. (A) Experimental overview. (B) GI transit time, (C) rectal bead expulsion time, (D) fecal pellet output and (E) fecal water content were measured to determine the GI motility in 'Young', 'Old' and 'Old + AGE' mice. (F) No significant difference in food intake was observed in each group. Results are shown as mean ± SEM, n=6/group. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. AGE, aged garlic extract; GI, gastrointestinal.

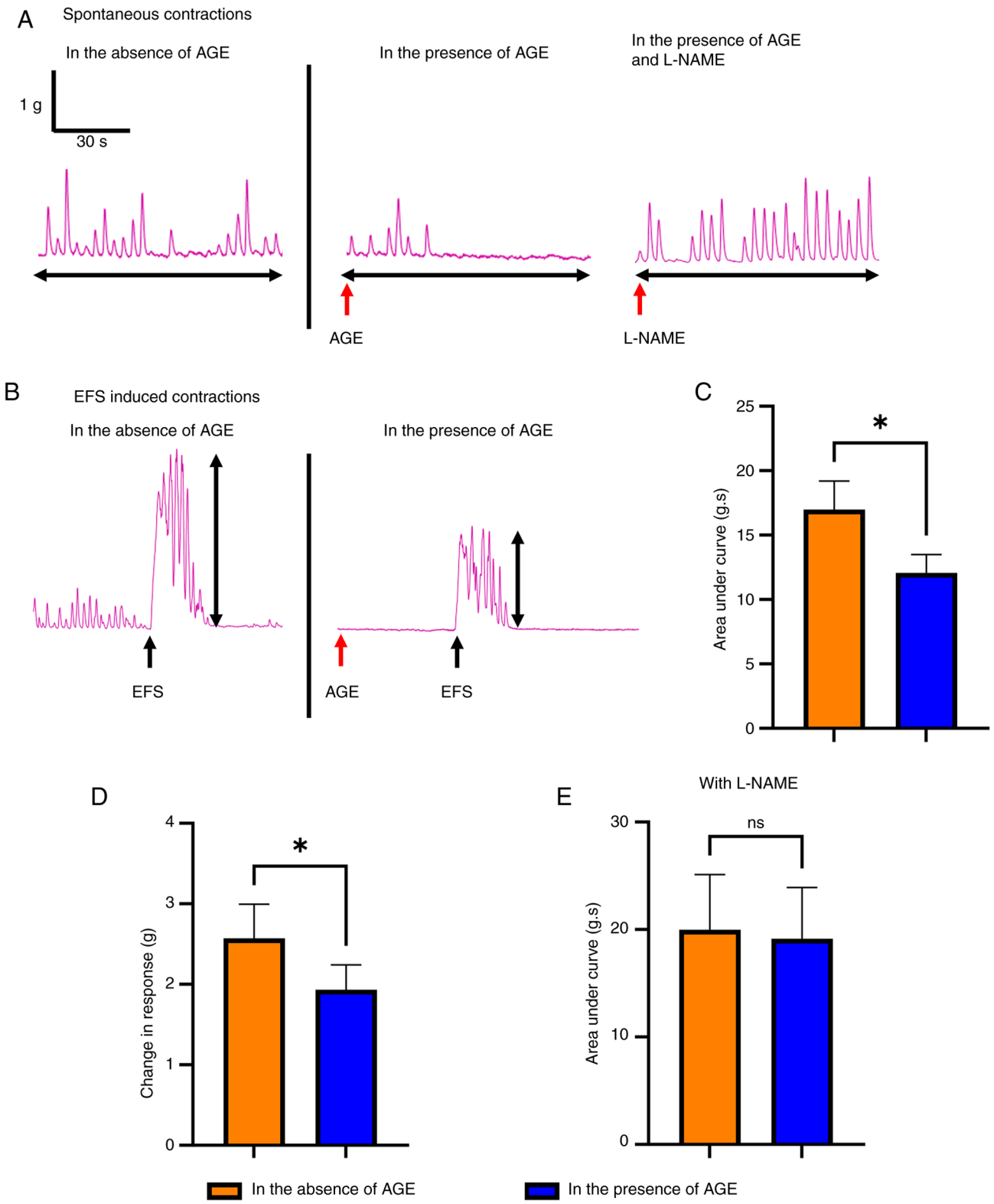

Figure 2. Effects of AGE on colonic smooth muscle activity. Representative traces of contractile activities of colonic smooth muscle in (A) spontaneous (double head arrows indicate range in which area under curve was quantified) and (B) in response to EFS (double head arrows indicate quantified maximum amplitude). Red and black arrows represent addiction of AGE or L-NAME, and application of EFS, respectively. (C) Area under curve (n=12/group) and (D) maximum amplitude of muscle contraction (n=7/group) were measured and compared between in the absence or presence of AGE. (E) L-NAME added to the organ bath abolished the effects of AGE (n=5/group). Results are shown as mean ± SEM. *P<0.05. EFS, electric field stimulation; L-NAME, NG-Nitroarginine methyl ester; AGE, aged garlic extract.

glia/progenitors (Fig. 6N, 156±9.4 in control vs. 63.2±4.6 in H_2O_2, P<0.0001). We also found a significant reduction in the length of neurites (Fig. 6O, 50.0±2.7 in control vs. 4.67±0.9 in H_2O_2, P<0.0001) and decreased cell viability (Fig. 6P, 100±11 in control vs. 46.3±7.9 in H_2O_2, P<0.01) in H_2O_2-treated ENS cells, confirming that oxidative stress

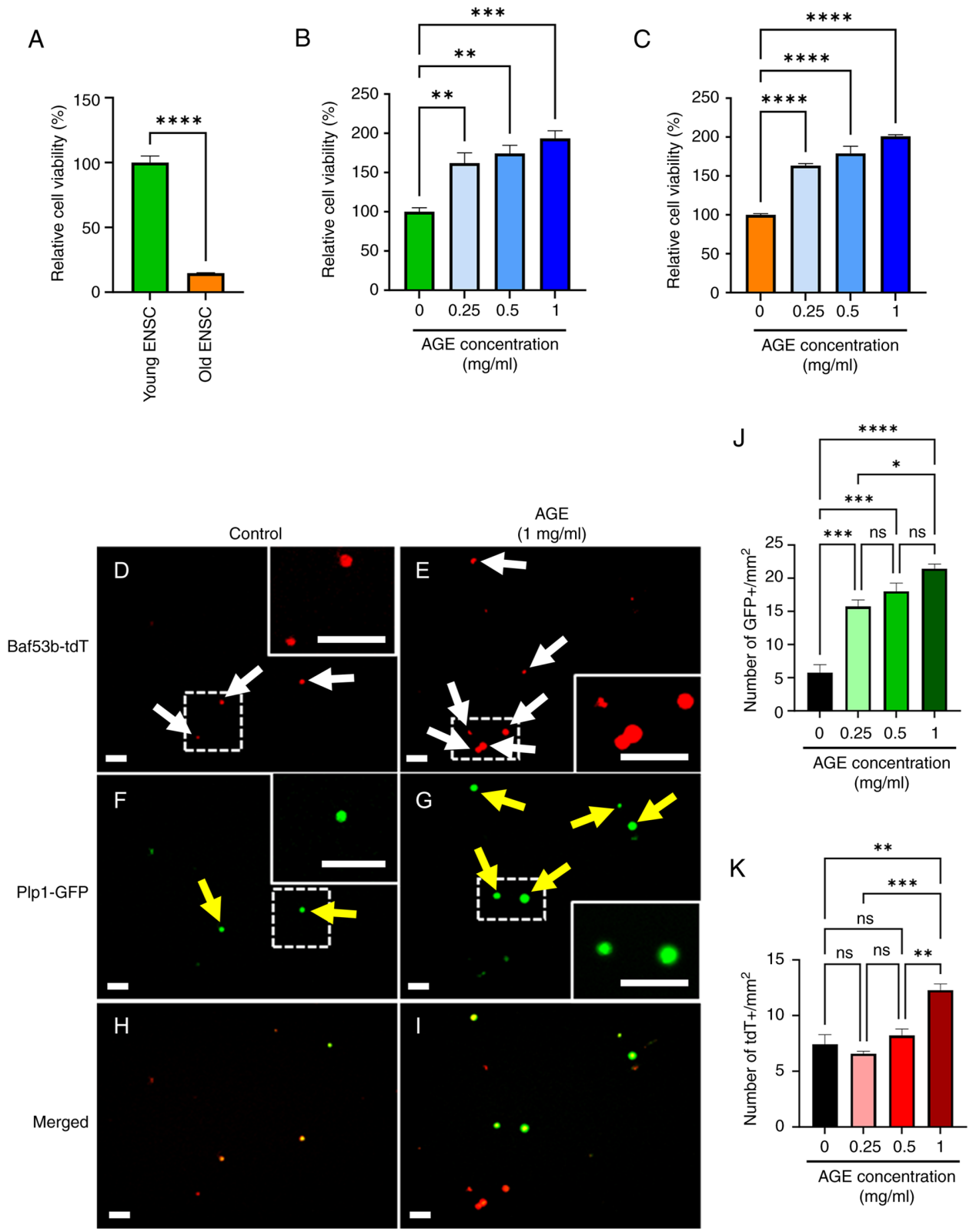

Figure 3. Effects of AGE on cultured ENS cells. (A) Significant reduction in viability of cells isolated from 'Old' mice. AGE improved viability of (B) 'Young'- or (C) 'Old'-derived ENSCs. (D-I) ENS cells were isolated from Plp1^{GFP}; Baf53b-tdT mice. (F, G and J) AGE increased the number of Plp1-GFP positive glia/neural progenitors (yellow arrows) and (D, E and K) Baf-tdT positive neurons (white arrows), respectively (dashed box enlarged in inset). Scale bars, 50 μm; magnification, x20. Results are shown as mean ± SEM, n=3/group. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. AGE, aged garlic extract; ENS, enteric nervous system; ENSC, enteric neural stem cells.

elicits damage to ENS cells in culture. When AGE was added to the H_2O_2-treated ENS cells at 0.5 mg/ml (Fig. 6G-I) and 1 mg/ml (Fig. 6J-L), there was significant improvement in the survival and health of neurons and glia. These findings suggest that AGE possesses neuroprotective effects on ENS cells against oxidative stress.

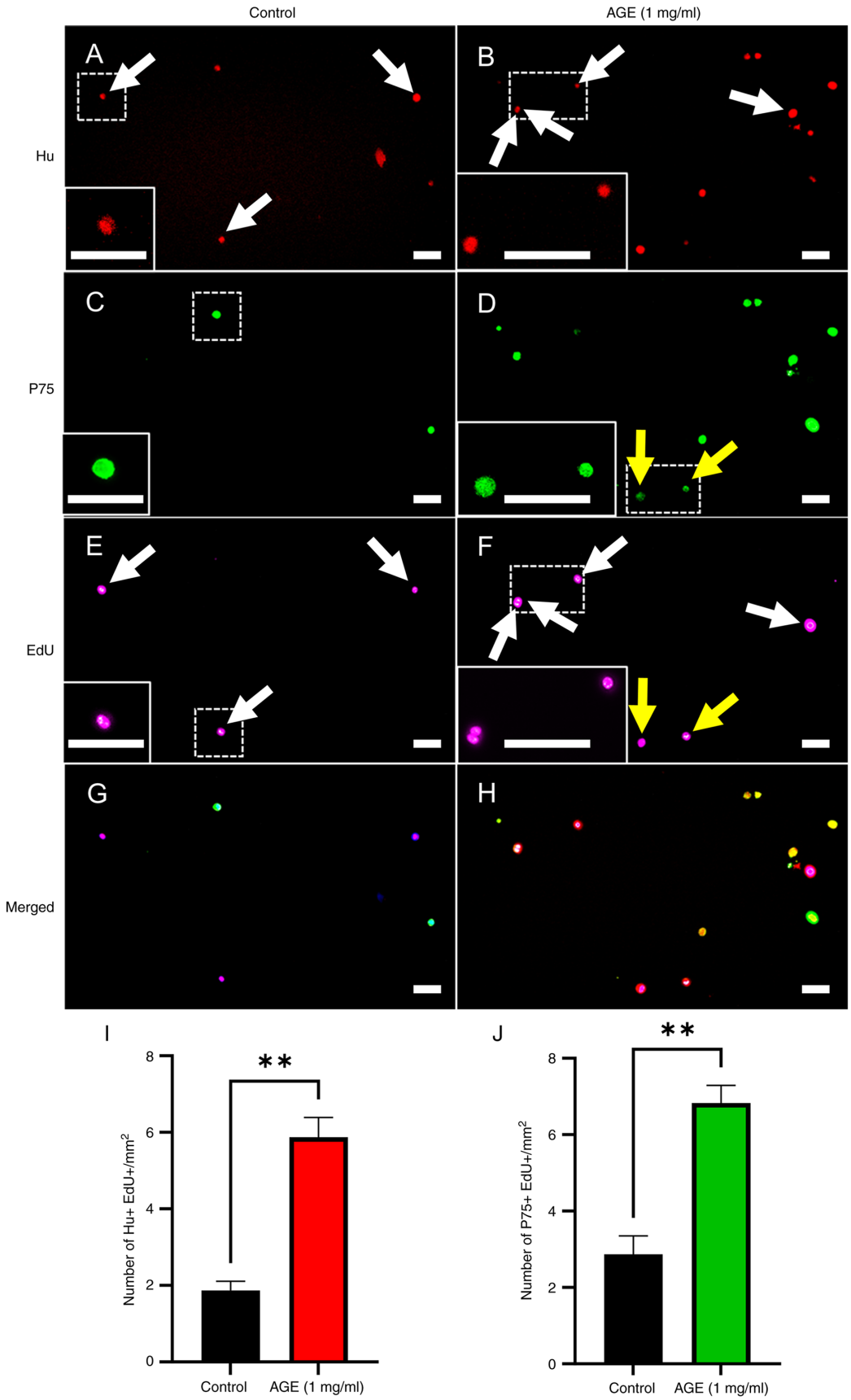

Figure 4. Effects of AGE on proliferation of cultured enteric neural cells. Cultured enteric neural cells were treated with PBS as vehicle control and immunostained for (A) Hu (neuron), (C) P75 (glia/neural progenitor cell) and (E) EdU, with the merged image shown in (G), (dashed box enlarged in inset). Treatment of 1 mg/ml AGE for cultured enteric neural cells was performed, followed by immunocytochemistry for (B) Hu, (D) P75 and (F) EdU, with the merged image presented in (H) (dashed boxes indicate enlarged insets). White arrows indcate Hu+/EdU+ neurons by treatment of (A and E) vehicle control and (B and F) 1 mg/ml AGE, respectively. (D and F) P75+/EdU+ cells were marked by yellow arrows in presence of 1 mg/ml AGE. Both the number of (I) Hu+/EdU+ neurons and (J) P75+/EdU+ cells significantly increased in the presence of 1 mg/ml AGE. Scale bars, 50 μm; magnification, x20, Results are shown as mean ± SEM, n=3/group. **P<0.01. EdU; 5-ethynyl-2'-deoxyuridine; AGE, aged garlic extract.

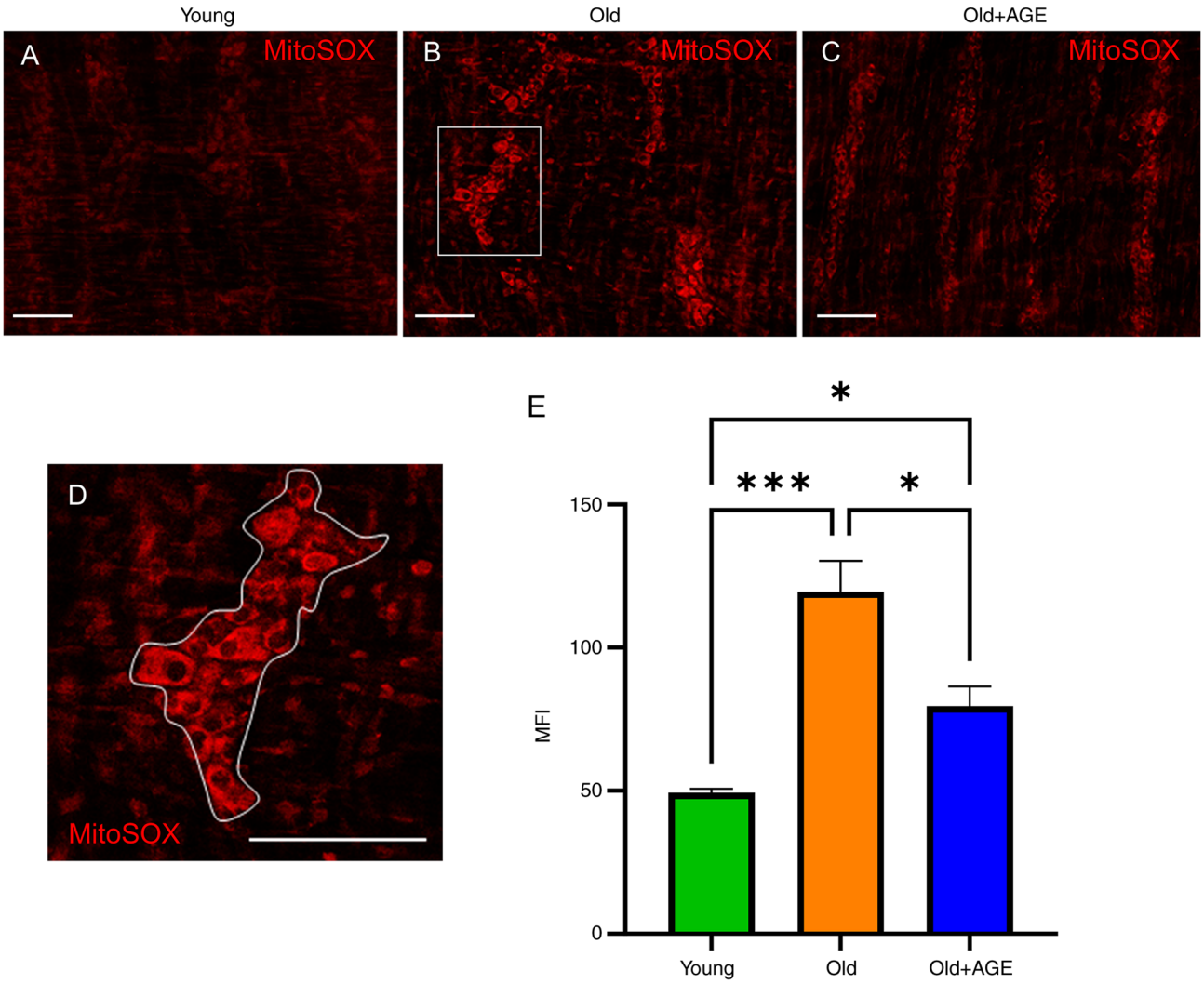

Figure 5. Effects of AGE on oxidative stress in myenteric plexus of old mice. MitoSOX staining of colonic myenteric layer of (A) 'Young' and (B) 'Old' and (C) 'Old + AGE' mice. (D) Enlarged image of white box is shown in (B). (E) AGE-fed old mice showed significantly less ROS in myenteric layer. Scale bars, 100 μm; magnification, x20. Results are shown as mean ± SEM, n=4/group. *P<0.05, ***P<0.001. ROS, reactive oxygen species; MFI, mean fluorescence intensity; AGE, aged garlic extract.

Discussion

In this study we examined the effects of AGE on colonic smooth muscle and ENS cells in old mice and demonstrated several positive effects on GI motility via colonic smooth muscle relaxation, enhancement of proliferation in enteric neural cells, and neuroprotective effects by reduction in oxidative stress. Our findings provide new insights for the treatment of aging-related GI dysmotility through use of a food supplement. The ENS is an extensive network of neurons and glia within the wall of the GI tract. The ENS regulates a variety of functions, including intestinal motility, sensation, absorption, secretion, and immunity (16). Abnormalities of the ENS, which can result from a number of different conditions, including age-associated neurodegeneration, lead to serious morbidity and reduced quality of life. Aging-related GI motility disorders are common, and include esophageal reflux, dysphagia, chronic constipation, rectal prolapse, and fecal incontinence. These contribute to the development of subsequent undernutrition, immunosuppression, sarcopenia, and frailty. GI disorders including irritable bowel syndrome and constipation are more prevalent in women than men, and several GI disorders increase with age (9,10). Therefore, we used aged female mice in this study. The mechanisms underlying aging-associated GI dysfunction are not fully understood, although a number of studies have shown that neuronal loss and/or molecular changes in the ENS may be involved (20,75,76). In the current study, we used 18-month-old mice as 'old' mice to evaluate i) gut motility using *in vivo* and *ex vivo* assays, ii) neurodegenerative ENS phenotype based on the degree of oxidative stress, and iii) properties of enteric neuronal stem cells (ENSCs) in culture. Consistent with previous reports (56,75), our study shows that both total gut transit time and rectal bead expulsion time are significantly delayed in old mice, and fecal pellet output and fecal water content are significantly reduced. We also observed significantly increased oxidative stress and a reduction in the proliferative capacity of ENSCs from old mice grown in culture, consistent with prior studies (69,77,78).

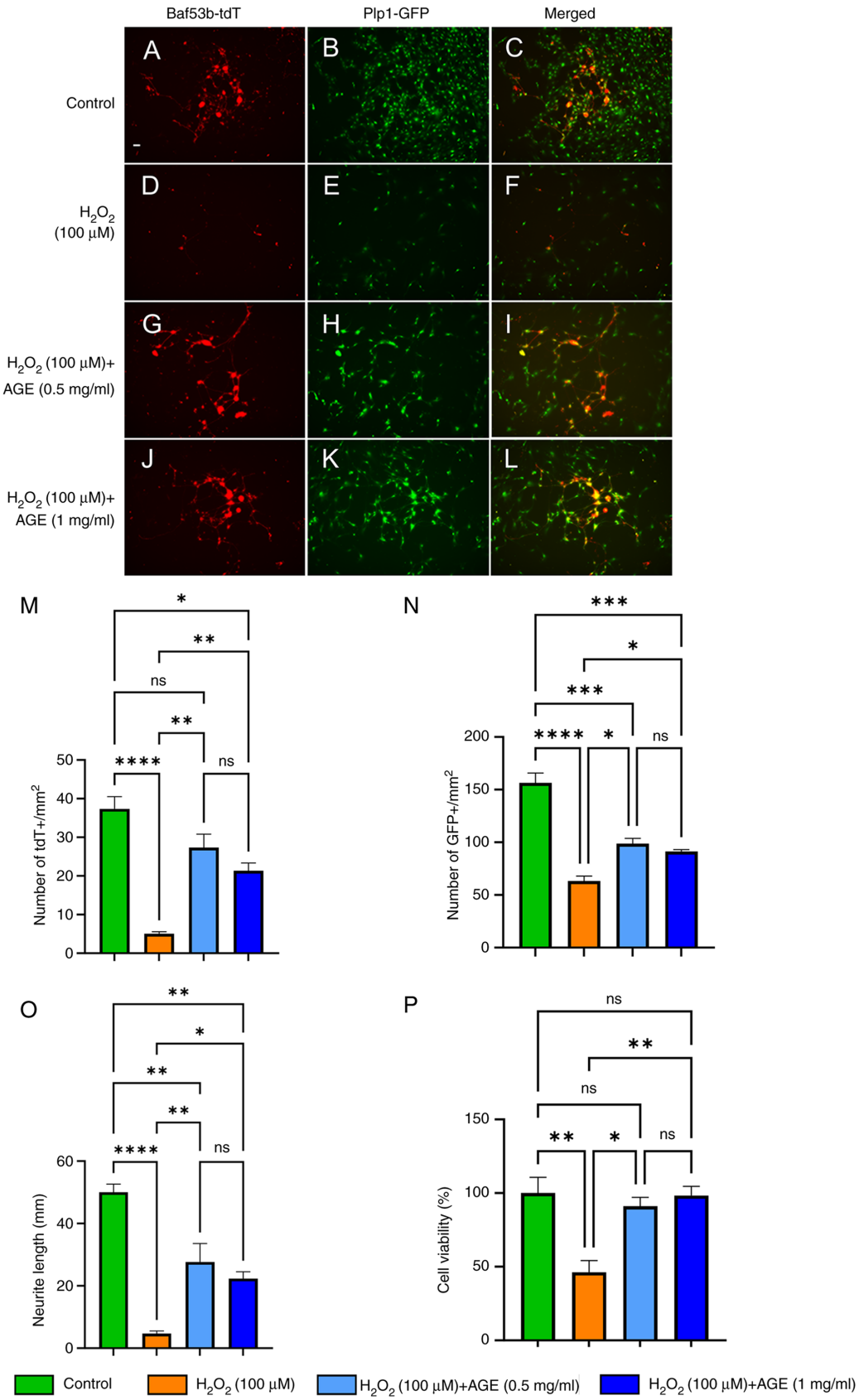

Figure 6. Neuroprotective effects of AGE on oxidative stress-induced enteric neural cells in culture. (A and B) Fluorescence images showed neurons and glial/neural progenitor cells labeled with Baf53b-tdT and Plp1GFP, respectively in the presence of PBS as a vehicle control, exhibiting normal morphology, and (C) is the merged image of (A) and (B). (D) The tdT-labeled neurons exposed to 100 μM hydroperoxide display neuronal damage. (E) GFP-labeled glial/neural progenitor cells appeared to a reduction of cell number, and (F) is the merged image of (D) and (E). The tdT-labeled neurons and GFP-labeled glial/neural progenitor cells were co-treated with 100 μM hydroperoxide and (G and H) 0.5 mg/ml or (J and K) 1 mg/ml AGE, which exhibited neuroprotective effects. (I) Presents the merged image of (G) and (H), and (L) presents the combined image of (J) and (K). The (M) number of neurons and (N) glial/neural progenitors, (O) neurite length and (P) cell viability were measured. Scale bars, 50 μm; magnification, x10. Results are shown as mean ± SEM, n=3/group. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. AGE, aged garlic extract.

AGE is a nutritional supplement prepared by prolonged extraction (normally over 10 months) of fresh garlic with 15-20% aqueous ethanol at room temperature (52). This product is odorless and appears to be superior to normal garlic in its antioxidant properties (79). It has been shown that AGE reduces total serum cholesterol and systolic pressure in hypercholesterolemic patients (27,80,81). AGE was further shown to promote antioxidant protection in cells by enhancing activity of the cellular antioxidant enzymes (35,79). Furthermore, recent work has demonstrated AGE-related neuroprotective effects against oxidative stress during neuroinflammation (79), leading us to hypothesize that AGE could have similar neuroprotective effects on the ENS and thus ameliorate the symptoms of GI dysmotility associated with aging.

Oxidative stress is characterized by an imbalance between enhanced production of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2) and superoxide anion (O_2-), and/or reduced antioxidant defenses due to mitochondrial dysfunction and a decline in antioxidant defenses with aging (82,83). This imbalance leads to lipid peroxidation and oxidation of proteins and DNA, leading to neurodegeneration (84,85). Elevated ROS levels in myenteric neurons in aged mice are associated with neuronal apoptosis (69), and dysmotility has been described in aganglionic mouse models (58,86) and old mice (56,75). Consistent with these previous reports, we observed significant elevation of ROS in the muscular layer of colon in old mice compared to young. Interestingly, these changes were ameliorated by an AGE-formulated diet for 2 weeks. It has been shown that AGE and its constituents, such as S-allylcysteine (SAC) and S-allylmercaptocysteine (SAMC), contain antioxidant properties (87). SAC is known to activate Nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway, resulting in enhancement of antioxidant defense (22,88). Furthermore, some antioxidants, including N-acetylcysteine (89), Vitamin C (90), and Resveratrol (91), have been shown to have neuroprotective properties. The current study, using H_2O_2-exposed neural cells to induce neurodegeneration *in vitro* (89), demonstrated a significant reduction in neuroglial damage in H_2O_2 treated cells in the presence of AGE. Therefore, these neuroprotective effects via reduction of ROS could be a mechanism explaining how AGE treatment improves aging-related colorectal dysmotility. Further studies investigating how AGE promotes ENS cell proliferation and reduces oxidative stress could explore the effects of AGE and its components on Nrf2 signaling including Nrf2, and its target genes such as HO-1 and GCLC (92,93).

The ENS is embryologically derived from the neural crest. During development, as undifferentiated NCCs migrate through the wall of GI tract, they differentiate into neurons and glial cells to form interconnecting enteric ganglia. A subpopulation of those enteric neural crest-derived cells remains undifferentiated and resides within enteric ganglia where they could act to replenish damaged or lost neurons in response to various insults, including inflammation, mechanical stretch, and aging (67,94,95). These cells, so-called enteric neuronal stem/progenitor cells (ENSCs), have been isolated from postnatal mice (60,62,96,97), swine, and humans and their capabilities, such as proliferation and migration, have been characterized following their culture *in vitro* (98-102). Kruger *et al* showed that in mice, the number of ENSCs declines more than 10-fold within the first 3 months of life. Moreover, their self-renewal capacity and neuronal differentiation potential decline by 50-60% (77). In our current study, we observed that the viability and the number of neurospheres generated by cultured ENSCs from old mice was significantly reduced compared to those from young mice. Interestingly, we found that the proliferative capacity of cultured ENSCs was enhanced by the addition of AGE. Although the mechanisms by which ENSC proliferation declines with age are not fully understood, a major chemical component of AGE, promotes the Nrf2 signaling pathway (22,88), which plays a key role in driving the cell cycle transition from G2 to M phase in hepatocytes (103) and also plays a role in regulating injury-induced neurogenesis in the brain (104). Based on our observed restoration in colorectal dysmotility in old mice treated with AGE, we hypothesize that improving an imbalance between contraction and relaxation in the gut could be beneficial for treatment of aging-related dysmotility. Since intestinal peristalsis consists of coordinated movements involving both contractions and relaxations (105), in support of this idea, colorectal dysmotility was described in nNOS KO mice (64) and reduction in the number of nNOS neurons was observed in old mice (76), suggesting that a reduction in (inhibitory) nNOS neurons results in a change in the ratios of excitatory and inhibitory enteric neurons, and that this imbalance may contribute to aging-related dysmotility. Further, AGE has been shown to have a relaxation effect in vascular smooth muscle by increasing nitric oxide production (106). In this current study, we observed that AGE significantly impacts colonic smooth muscle contractility, as both baseline contractile activity and electrically evoked responses in colonic muscle strips were reduced in the presence of AGE, findings that together highlight its potent modulatory influence on motility. The observed effects of AGE suggest that it increases muscle relaxation, as reduced contraction and enhanced relaxation are complementary aspects of muscle tone regulation (107). Furthermore, these effects of AGE were blocked by L-NAME, an inhibitor of nNOS, suggesting that nitric oxide (NO) plays an important role in mediating the muscle relaxant effects of AGE. NO is generated from L-Arginine as a substrate by nNOS, which promotes smooth muscle relaxation by activating guanylate cyclase and increasing cyclic GMP levels (108,109). L-Arginine is one of the components found in AGE, and as a main source of NO has a relaxation effect in vascular smooth muscle (106). The role of NO in mediating smooth muscle relaxation, particularly in the rodent colon, is well-established, and NO released from sodium nitroprusside induces relaxation of gastrointestinal smooth muscle (110-112). The observed inhibition of AGE's effects by L-NAME reinforces the involvement of the nNOS-NO pathway in mediating AGE's action (91). Thus NO derived from L-arginine contained in AGE may be inducing the relaxation effects that we observed in colonic smooth muscle, which could lead to improvement in aging-related dysmotility. This could be further investigated in future studies using nitrate/nitrite colorimetric assays as previously described (33,106), and molecular biological assays (western blotting, quantitative PCR) and/or biochemical assays could be used to evaluate the effects of AGE and its constituents on

downstream targets of nNOS signaling including guanylate cyclase, PKA/PKG and myosin light chain kinase (113).

Although not directly tested in our studies, another mechanism that could be involved in the observed effects of AGE on gut contractility is smooth muscle relaxation via hydrogen sulfide (H_2S) (114), and organic polysulfides as its potential source is contained in AGE (115-117). H_2S facilitates membrane hyperpolarization in smooth muscle cells, reducing their excitability and contractile responses. This effect may explain the observed attenuation in both spontaneous contractility and EFS-induced responses, but requires further investigation. The ability of H_2S to modulate ion channel activity and intracellular calcium dynamics further supports this hypothesis, as these processes are critical for smooth muscle contraction and neuromuscular signaling (118,119). These mechanisms highlight AGE's potential to counteract age-related decline in gut motility (60,88,90) by promoting smooth muscle relaxation. Studies on GI physiology have shown that interventions reducing contractility effectively promote muscle relaxation (107,120). Furthermore, it is unknown if the effects of AGE persist in the long term. Several previous studies have shown that gut microbiota can influence GI motility (121) and have a range of effects on the ENS (122). A human study demonstrated that garlic intake for one week improved gut microbial diversity and increased the relative abundance of beneficial bacteria including Faecalibacterium prausnitzii and Akkermansia spp (123), supporting the idea that changes induced in the short term could have long term implications. However, we recognize that determination of the prolonged impact of AGE on the gut is a limitation of our study. Nevertheless, our results provide valuable insight into the therapeutic potential of AGE for enhancing colonic motility through NO-dependent pathways, supporting previous observations of its effects on smooth muscle systems (124).

In conclusion, an AGE diet in mice resulted in improvements in aging-related colorectal dysmotility involving colonic smooth muscle relaxation via nNOS, enhancement of proliferation in enteric neural cells, and neuroprotective effects. Our findings provide both a characterization of motility in aged mice and reveal potential beneficial effects for GI motility disorders and ENS cells through AGE.

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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

KO and RH designed the experiments and are major contributors in writing the manuscript. KO performed *in vitro* study. KO, RH and TO preformed *in vivo* study. AAR performed organ bath study. KO, RH and AAR confirmed the authenticity of all the raw data. AJB and AMG supervised this study, contributed to the conception and data interpretation, and reviewed and edited the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

This study was conducted in accordance with the protocols reviewed and approved by the Institutional Animal Care and Use Committee at Massachusetts General Hospital (approval nos. 2009N000239 and 2013N000115). All methods were carried out in accordance with relevant guidelines and regulations.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have competing interests: the work was funded by Wakunaga Pharmaceutical Company Ltd., where KO is an employee.

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