Qing'E formula alleviates the aging process in D-galactose-induced aging mice

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Abstract. Qing'E formula (QEF) is a clinically used prescription with four ingredients, *Eucommiae Cortex*, *Psoraleae Fructus*, *Juglandis Semen* and *Garlic Rhizoma*, from the Song dynasty (10th century CE). The present study aimed to investigate the anti-aging effect and mechanisms of QEF on D-galactose-induced aging mice. A mouse subacute aging model was established by subcutaneous injection of D-galactose at the neck consecutively for 8 weeks. Motor activity and memory impairment of the mice were evaluated by the rotarod test and passive avoidance test, respectively. Serum and liver parameters were analyzed with biochemical kits. Hippocampal mRNA and protein expression levels were examined by reverse transcription-quantitative polymerase chain reaction and western blotting, respectively. QEF administration significantly ameliorated the impaired motor and memory of aging mice. In the serum, QEF reduced blood urea nitrogen, creatinine, nitric oxide (NO) and malondialdehyde (MDA) levels, and inhibited alanine aminotransferase and aspartate aminotransferase activities. In the liver, QEF increased the glutathione level, enhanced total antioxidant capacity and catalase activity, deceased NO and MDA production, and reduced NO synthase activity. In the hippocampus, QEF elevated gene expression levels of Klotho, sirtuin 1 (*SIRT1*), forkhead box transcription factor O3, peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), insulin-like growth factor-1 and peroxiredoxin-3. QEF increased protein expression levels of Klotho and SIRT1, and decreased that of PGC-1α in the hippocampus. In conclusion, QEF attenuated the aging process in D-galactose-treated mice, which may be mediated through enhancing the antioxidants in the body, protecting renal and hepatic health, and balancing hippocampal expression levels of the longevity-related genes.

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Abbreviations: QEF, Qing'E formula; D-gal, D-galactose; GSH, glutathione; CAT, catalase; T-AOC, total antioxidant capacity; MDA, malondialdehyde; NO, nitric oxide; NOS, nitric oxide synthase; BUN, urea nitrogen; CREA, creatinine; ALT, alanine aminotransferase; AST, aspartate aminotransferase; SIRT1, sirtuin 1; FOXO3, forkhead box O transcription factor 3; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α; IGF-1, insulin-like growth factor-1; PRDX-3, peroxiredoxin-3

Key words: D-galactose, aging, Qing'E formula, impaired memory, longevity

Introduction

Aging is a natural process that increases the vulnerability of an organism to the challenges of environment and diseases. For humans, the aging of a population implicates the rising morbidity and mortality of age-associated disorders, such as Alzheimer's disease, heart failure and stroke, which exert a heavy burden on society. As is known so far, >300 theories (1) of aging have been proposed, suggesting a complex and multifactorial process. For a long time, slowing or delaying the aging process has attracted increasing attention in the field of medicine research. However, there are no supplements or products that are known to stop the aging process (2).

Chronic D-galactose administration has been demonstrated to accelerate the aging process in rodents and *Drosophia* (3,4). The animals administered with D-galactose exhibited typical symptoms, such as cognitive dysfunction, neurodegeneration, weakened motor function and shortened lifespan that resemble the natural process of aging. Therefore, this model is used widely for the study of the aging process and screening of anti-aging drugs.

Qing'E formula (QEF) is a traditional prescription with four ingredients, *Eucommiae Cortex*, *Psoraleae Fructus*, *Juglandis Semen* and *Garlic Rhizoma*, in a specified ratio according to the Chinese Pharmacopoeia (5). In China, QEF was used 1,000 years ago from the Song dynasty (10th century CE), and it is associated with invigorating the kidneys, replenishing bone and muscle, causing the body to become slimmer and benefiting complexion. QEF can clinically

alleviate osteoporosis in postmenopausal woman (6), and improve menopausal symptoms (7). However, little is known regarding whether QEF has an anti-aging effect. In the present study, the anti-aging function of QEF was assessed on D-galactose-induced aging mice. QEF showed marked attenuation on the impaired motor and memory of mice. Following this, the possible underlying mechanisms were investigated. The study may contribute to the clinical application of QEF as a remedy for anti-aging.

Materials and methods

Materials. QEF was prepared as reported previously (8). Briefly, the four ingredients, *Eucommiae Cortex* (baked with salt), *Psoraleae Fructus* (baked with salt), *Juglandis Semen* and *Garlic Rhizoma* (steamed and dried) were mixed at the ratio of 16:8:5:4 (w/w) and pulverized to a fine powder following identification by Professor Hong Xu, at the Ministry of Education Key Laboratory for Standardization of Chinese Medicines, Shanghai University of Traditional Chinese Medicine (Shanghai, China). Specimens of the herbs were kept in the laboratory for authentication. The representative components of QEF determined by high-pressure liquid chromatography were as follows: Psoralen (0.0785%), isopsoralen (0.0668%), isobavachalcone (0.098%), bavachin (0.031%), corylifol A (0.036%), neobavaisoflavone (0.056%) and pinoresinol diglucoside (0.024%).

Biochemical kits for measuring glutathione (GSH), catalase (CAT), total antioxidant capacity (T-AOC), total superoxide dismutase (T-SOD), malondialdehyde (MDA), nitric oxide (NO), NO synthase (NOS), blood urea nitrogen (BUN), creatinine (CREA), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). D-galactose was obtained from Sigma-Aldrich (St. Louis, MO, USA). All the other reagents were of analytical grade and commercially available.

Animals and treatment. Three-month-old male mice (Kunming strain), weighing 45±5.0 g, were provided by Shanghai University of Traditional Chinese Medicine. The animals were acclimated to the laboratory environment for 1 week before the experiment. They were housed under standard conditions (25˚C; 12-h light and 12-h dark) with free access to food and water. All experimental procedures were carried out in compliance with the Chinese legislation on the use and care of laboratory animals and were approved by the university committees for animal experiments.

The mice were divided randomly into 6 groups (12 mice/group): The control, model, vitamin E (VE), and QEF-low, -middle and -high groups. Mice in the control group were intraperitoneally injected with saline, and the other mice were administered a daily subcutaneous injection of D-galactose (100 mg/kg/day) at the neck consecutively for 8 weeks. Mice also received intragastric gavage with different drugs suspended in 0.5% sodium carboxymethyl cellulose. The control and model group mice were treated with saline. For VE group mice, they were administered with VE (100 mg/kg/day). And for QEF group mice, they were given QEF at doses of 1.62 (QEF-L), 3.24 (QEF-M) or 6.48 (QEF-H) g/kg/day.

Behavioral tests

Rotarod test (RRT). RRT was carried out with a Rota-Rod Treadmill (Mobiledatum, Inc., Shanghai, China). Subsequent to being trained to walk steadily on a horizontally oriented rod, the mice walked on the rod rotating at 40 rpm for a ≤600 sec on the testing day. The trial for each mouse was repeated three times with an interval longer >1 h. The data were averaged for statistical analysis.

Passive avoidance test (PAT). PAT was conducted in the shuttle boxes, as described previously (9). Briefly, on day 1, the mice were trained to learn to stay in the bright chamber with a criterion of 300 sec. On day 2, the time for the passive avoidance response of the animals was evaluated in 300 sec. The latency to enter the dark chamber and the number of entries were recorded by a video-tracking system (Mobiledatum, Inc.).

Biochemical analysis. Following behavioral tests, the mice were anesthetized with isoflurane. The orbital blood of mice was collected. Subsequently, the liver, kidney and hippocampus from the mice were dissected on ice. All the tissue samples were snap-frozen in liquid nitrogen and stored at -80°C until further analysis. The activities of T-AOC, ALT, AST, CAT, T-SOD and NOS, and the levels of GSH, MDA, NO, BUN and CREA were determined using the respective kits, following the manufacturer's protocols.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNAs from hippocampal tissues of mice were extracted using TRIzol according to the manufacturer's protocol (Life Technologies, Thermo Fisher Scientific, Inc., Waltham, MA, USA). Subsequent to eliminating the trace amount of DNA contamination with DNase I, the RNA was reverse transcribed into cDNA with the RevertAid First Strand cDNA Synthesis kit (Fermentas, Thermo Fisher Scientific, Inc.). The synthesized cDNA was used as templates for the RT-qPCR reaction using the Taqman SYBR kit (Life Technologies, Thermo Fisher Scientific, Inc.). The primers used [Klotho, sirtuin 1 (SIRT1), forkhead box O transcription factor 3 (FOXO3), peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), insulin-like growth factor-1 (IGF-1) and peroxiredoxin-3 (PRDX-3)] are listed in Table I. Relative expression levels of the respective genes were normalized to that of glyceraldehyde-3-phosphate dehydrogenase within the same sample.

Western blotting. Hippocampal tissues were homogenized by sonication in CelLytic™ MT mammalian tissue lysis reagent (Sigma-Aldrich) supplemented with protease and phosphatase inhibitor cocktails. After centrifugation at 13,523 x g at 4˚C for 10 min, the supernatant of the lysate was collected and subjected to SDS-PAGE. Following this, the proteins were transferred onto polyvinylidene fluoride membranes. Subsequent to blocking with 5% skimmed milk, the membranes were incubated with primary antibodies against SIRT1 (cat. no. ab110304; 1:1,000, mouse monoclonal antibody), PGC-1α (cat. no. ST1202; 1:1,000, mouse monoclonal antibody), KLOTHO (cat. no. ab154163; 1:1,000, rabbit monoclonal antibody) and β-actin (cat. no. 4967; 1:5,000, rabbit polyclonal antibody) overnight at 4˚C followed by incubation with respective horseradish peroxide-conjugated secondary antibodies. The bands were visualized with the ECL

Table I. Primer sequences for reverse transcription-quantitative polymerase chain reaction.

Gene	Forward primer	Reverse primer
GAPDH	ATGTGTCCGTCGTGGATCTGA	ATGCCTGCTTCACCACCTTCT
Klotho	GGGACACTTTCACCCATCACT	ACGTTGTTGTAACTATCGCTGG
SIRT1	GCTGACGACTTCGACGACG	TCGGTCAACAGGAGGTTGTCT
PGC-1α	TATGGAGTGACATAGAGTGTGCT	CCACTTCAATCCACCCAGAAAG
PRDX-3	TCGACGACTTTAAGGGGAAA	CCATTCTTTCTTGGCGTGTT
IGF-1	GATGGGGAAAATCAGCAGTC	GCTGGTAAAGGTGAGCAAGC
FOXO3	CTGGGGGAACCTGTCCTATG	TCATTCTGAACGCGCATGAAG

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SIRT1, sirtuin 1; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α; PRDX-3, peroxiredoxin-3; IGF-1, insulin-like growth factor-1; FOXO3, forkhead box O transcription factor 3.

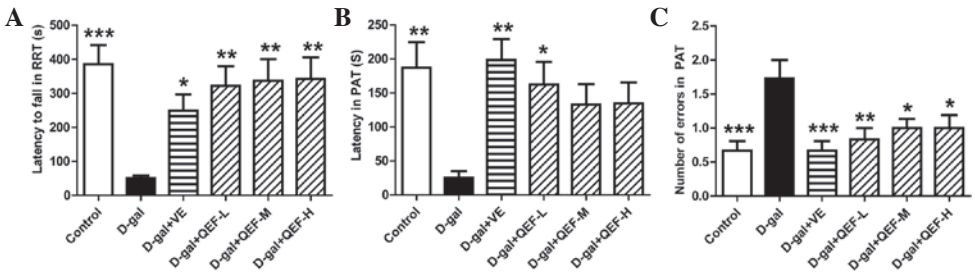

Figure 1. Effect of QEF on D-gal-induced aging mice behavior. (A) QEF prolonged residence time of aging mice on rod in RRT. (B) QEF increased latency to the dark chamber of aging mice in PAT. (C) QEF decreased the erroneous entries of aging mice in PAT. All the data are presented as mean ± standard error of the mean. n=10-12/group. *P<0.05; **P<0.01; ***P<0.001 vs. D-gal group. QEF, Qing'E formula; D-gal, D-galactose; L, low dose (1.62 g/kg/day); M, medium dose (3.24 g/kg/day); H, high dose (6.48 g/kg/day); VE, vitamin E; RRT, rotarod test; PAT, passive avoidance test.

prime kit (GE Healthcare, Chalfont, UK). Relative quantification of the blots was conducted using ImageJ 1.46r (National Institute of Health, Bethesda, MD, USA).

Statistical analysis. All data are presented as mean ± standard error of the mean. The difference between groups was evaluated by one-way analysis of variance with Dunnett's multiple comparison test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of QEF on behavioral parameters. Following treatment with QEF for 8 weeks, the mice were first subjected to RRT. As shown in Fig. 1A, D-galactose-injected mice fell quicker from the rod in the RRT (P<0.001) in comparison with the control group mice. Similar to VE, the positive drug, QEF at all doses significantly prolonged the residence time of mice on the rod (P<0.01).

In PAT, D-galactose injection impaired the memory of mice as they exhibited shorter latency (Fig. 1B) (P<0.01) but increased entries (Fig. 1C) (P<0.001) into the dark chamber. Although only low dose of QEF significantly extended the latency of D-galactose-treated mice (P<0.05), all doses of QEF on mice markedly reduced their entries into the dark chamber.

The behavioral tests indicated that QEF treatment could enhance motor coordination and alleviate the impairment of memory of the aging mice.

Effect of QEF on serum parameters. Biochemical tests showed that D-galactose increased the serum levels of BUN, CREA, AST, ALT, NO and MDA markedly (P<0.05 or P<0.001) (Fig. 2). QEF treatment at all doses could reverse all the aforementioned indexes, although not in a dose-dependent manner (P<0.05, P<0.01 or P<0.001). By contrast, VE could only decrease serum BUN, CREA and AST levels (P<0.05 or P<0.001). The results implicated that QEF attenuated the aging process in the blood.

Effect of QEF on liver parameters. In the mouse liver, long-term D-galactose treatment decreased GSH level and reduced T-AOC and CAT activities (P<0.05 or P<0.001) (Fig. 3A-C). However, it induced the elevation of NO, MDA levels and NOS activity (P<0.05 or P<0.01) (Fig. 3D-F). Following QEF treatment, all the biochemical parameters in liver were improved. However, the positive control, VE, improved GSH, MDA and NOS in the liver (P<0.05 or P<0.01). These suggested that QEF improved the antioxidative system in liver.

Effect of QEF on the hippocampal parameters. In the hippocampus, D-galactose did not show significant changes in mRNA expression levels of Klotho, SIRT1, FOXO3, PGC-1α, IGF-1 and PRDX-3 (Fig. 4), the genes that are well known to be actively involved in aging process. However, QEF treatment for 8 weeks could elicit significant upregulation of these genes (P<0.05, P<0.01 or P<0.001), which appeared to be in a dose-dependent manner. By contrast, VE treatment only enhanced the gene expression of Klotho (P<0.01). Consistent

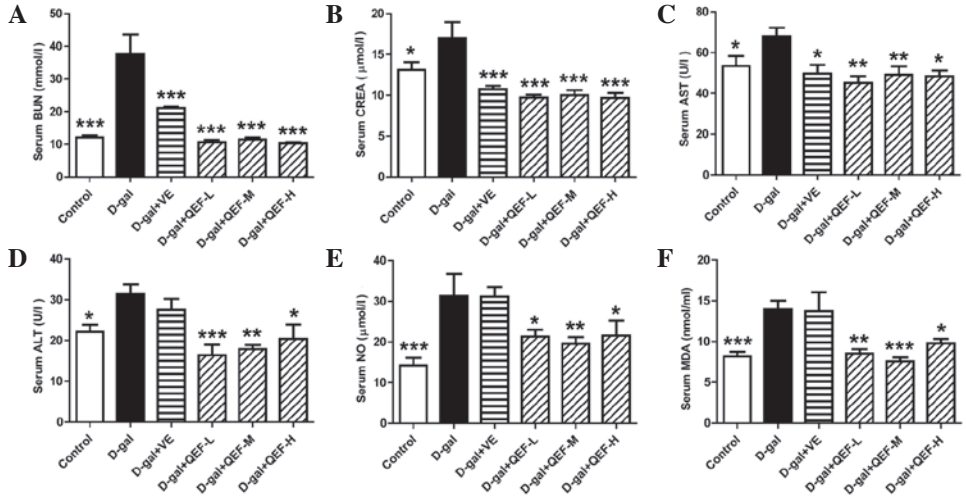

Figure 2. Effect of QEF on the serum parameters of D-gal-induced aging mice. (A) QEF improved the increased BUN level. (B) QEF prevented the elevation of CREA level. (C) QEF reduced the increased AST activity. (D) QEF deceased the increased ALT activity. (E) QEF lessened the elevated NO level. (F) QEF mitigated the increased serum MDA level. All data are presented as mean ± standard error of the mean. n=10-12/group. *P<0.05, **P<0.01, ***P<0.001 vs. D-gal group. QEF, Qing'E formula; D-gal, D-galactose; L, low dose (1.62 g/kg/day); M, medium dose (3.24 g/kg/day); H, high dose (6.48 g/kg/day); VE, vitamin E; BUN, blood urea nitrogen; CREA, creatinine; AST, aspartate aminotransferase; ALT, alanine aminotransferase; NO, nitric oxide; NOS, NO synthase.

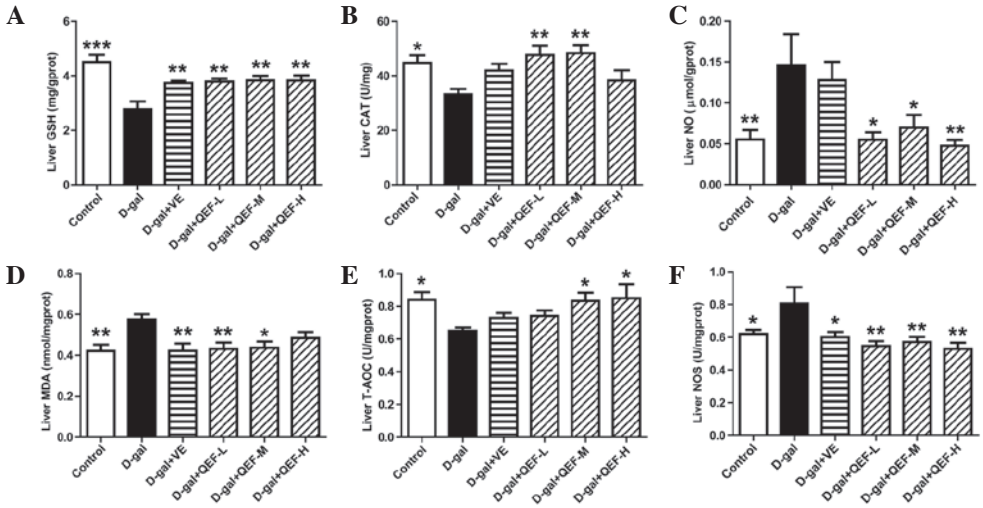

Figure 3. Effect of QEF on liver parameters of D-gal-induced aging mice. (A) QEF increased GSH level. (B) QEF elevated CAT activity. (C) QEF decreased the elevated NO level. (D) QEF reduced the production of MDA. (E) QEF enhanced T-AOC. (F) QEF inhibited the NOS activity. All data are presented as mean ± standard error of the mean. n=10-12/group. *P<0.05, **P<0.01, ***P<0.001 vs. D-gal group. QEF, Qing'E formula; D-gal, D-galactose; L, low dose (1.62 g/kg/day); M, medium dose (3.24 g/kg/day); H, high dose (6.48 g/kg/day); VE, vitamin E; GSH, glutathione; T-AOC, total antioxidant capacity; CAT, catalase; NO, nitric oxide; MDA, malondialdehyde; NOS, NO synthase.

with its effect on mRNA expression, QEF treatment induced significant expression of Klotho and SIRT1 at protein levels (P<0.05, P<0.01 or P<0.001) (Fig. 5A-C). In terms of PGC-1α, D-galactose induced marked elevation of the protein (P<0.001), the effect of which could be counteracted by QEF treatment (P<0.01 or P<0.001) (Fig. 5D). For VE, it increased Klotho and SIRT1 levels, but reduced the PGC-1α level (P<0.05, P<0.01 or P<0.001). These results implicated that QEF administration improved the aging process in hippocampus.

Discussion

In the present study, the effect of QEF on the D-galactose-induced aging mice was evaluated. The results showed that QEF improved motor coordination and memory impairment in aging mice, indicating its potential application as an anti-aging drug.

As aging occurs, the body loses muscle component, resulting in a loss of strength and more easily fatigued muscle (10), which is accompanied with declined learning and memory capability. In the present study, the motor coordination of mice reflecting one aspect of the strength of muscle was evaluated by RRT (11), while the memory of mice was assessed with PAT (12). In the experiments, D-galactose-treated mice showed typical aging symptoms, as evidenced by reduced endurance in RRT and impaired memory in PAT. By contrast, QEF treatment could reverse the deteriorated behavior of mice, suggesting its alleviative effect on motor and memory of aging mice.

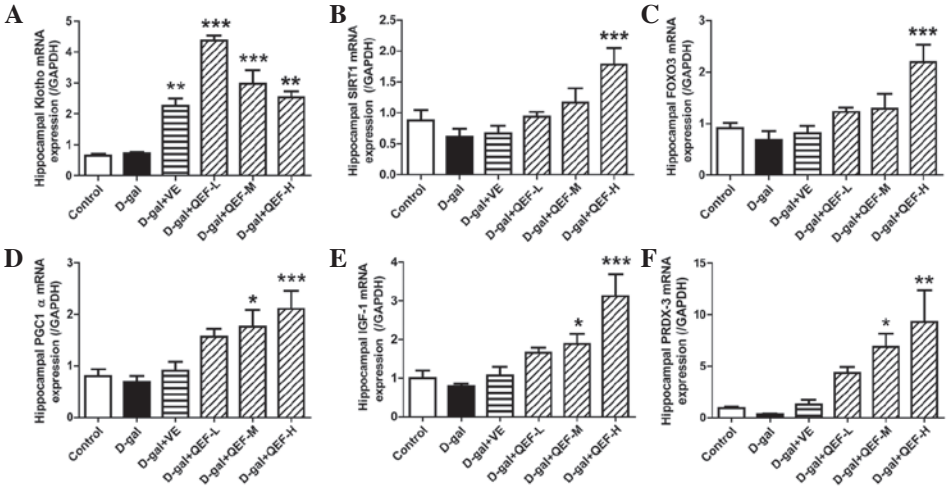

Figure 4. Effect of QEF on hippocampal gene expression levels of D-gal-induced aging mice. QEF increased (A) Klotho, (B) SIRT1, (C) FOXO3, (D) PGC-1α, (E) IGF-1 and (F) PRDX-3 mRNA expression levels. All data are presented as mean ± standard error of the mean. n=4-5/group. *P<0.05, **P<0.01, ***P<0.001 vs. D-gal group. QEF, Qing'E formula; D-gal, D-galactose; L, low dose (1.62 g/kg/day); M, medium dose (3.24 g/kg/day); H, high dose (6.48 g/kg/day); VE, vitamin E; SIRT1, sirtuin 1; FOXO3, forkhead box O transcription factor 3; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α; IGF-1, insulin-like growth factor-1; PRDX-3, peroxiredoxin-3.

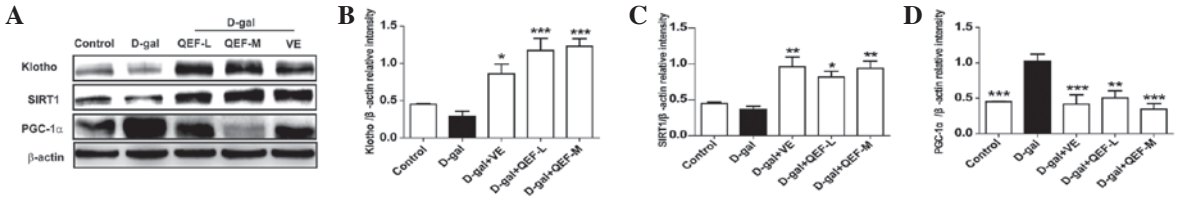

Figure 5. Effect of QEF on hippocampal protein expressions of D-gal-induced aging mice. (A) Western blot analysis. Gray intensity analysis of (B) Klotho, (C) SIRT1 and (D) PGC-1α. All data are presented as mean ± standard error of the mean. n=4/group. *P<0.05, **P<0.01, ***P<0.001 vs. D-gal group. QEF, Qing'E formula; D-gal, D-galactose; L, low dose (1.62 g/kg/day); M, medium dose (3.24 g/kg/day); H, high dose (6.48 g/kg/day); VE, vitamin E; SIRT1, sirtuin 1; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α.

The free radical theory is one of the most popular aging theories. According to the theory, accumulated free radicals, such as reactive oxygen species (ROS), damage lipids, DNA, proteins and tissues in organisms (13). Enzymatic antioxidants, such as superoxide dismutase, CAT and GSH peroxidase, and non-enzymatic antioxidants, such as VE and GSH, can neutralize ROS and prevent against further injury. In the present study, QEF administration enhanced antioxidants, including GSH, T-AOC and CAT, in the serum or liver of D-galactose-induced aging mice. As a result, MDA, the product of lipid peroxidation and marker for oxidative stress (14), was reduced. These results demonstrated the regulatory effect of QEF on the unbalanced oxidants in aging mice.

Klotho, or its secreted form, α Klotho, has been demonstrated to function as an anti-aging and organ protection factor by inhibiting signaling of multiple growth factors, such as insulin, IGF-1 and TGF-β (15-17). SIRT1 is a key protein that controls the aging process by the regulation of energy metabolism (18), and has been shown to benefit the health of aging mice (19). FOXO3 is a potent transcriptional activator that triggers the expression levels of numerous genes involved in cell cycle arrest, DNA repair, hypoxia and apoptosis (20). In humans, single-nucleotide polymorphisms of *FOXO3* have been shown to be closely associated with longevity (21) and have an important role in ameliorating senescence and aging (22). PRDX-3 is mainly responsible for the detoxification of 90% of the hydrogen peroxide in the mitochondria (23). By contrast, low IGF-1 signaling is closely associated with improved longevity (24). As aforementioned, QEF improved impaired memory of D-galactose-treated mice, therefore, the expression levels of these genes or their products in hippocampus were investigated. The present results suggested that QEF alleviated memory impairment was associated with balancing the expression levels of longevity-related genes at mRNA or protein levels.

Blood BUN and CREA are the common indicators for kidney health, while ALT and AST are the common parameters for the evaluation of liver health. In the present study, chronic D-galactose administration was shown to increase all the serum parameters significantly. By contrast, QEF treatment could efficiently prevent against the elevation of BUN, CREA, ALT and AST, implicating a protective effect on the liver and kidney. However, its mechanism remains to be elucidated at the current stage and requires further investigation.

In conclusion, QEF counteracted the accelerated aging process induced by D-galactose in mice, which may be due to its effects on enhancing antioxidants, protecting renal and hepatic health, and balancing hippocampal expression levels of the longevity-related genes.

Acknowledgements

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