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Effect of quercetin on chronic enhancement of spatial learning and memory of mice

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Abstract In this study we evaluated the effect of quercetin on *D*-galactose-induced aged mice using the Morris water maze (MWM) test. Based on the free radical theory of aging, experiments were performed to study the possible biochemical mechanisms of glutathione (GSH) level and hydroxyl radical (OH^-) in the hippocampus and cerebral cortex and the brain tissue enzyme activity of the mice. The results indicated that quercetin can enhance the exploratory behavior, spatial learning and memory of the mice. The effects relate with enhancing the brain functions and inhibiting oxidative stress by quercetin, and relate with increasing the GSH level and decreasing the OH^- content. These findings suggest that quercetin can work as a possible natural anti-aging pharmaceutical product.

Keywords: quercetin, aging, learning and memory, free radical, reactive oxygen species (ROS), glutathione (GSH)

Quercetin belongs to a family of plant pigments called flavonoids that are a ubiquitous group of polyphenolic substances which are present in most plants, extensively existing in vegetables, fruits, e.g. onions, potatoes, cabbages, lettuces, apples, mangoes and black currants. A great number of plant medicines contain flavonoids, which have been reported by many authors as having developing coronary vessel, reducing blood fat, anti-thrombotic, anti-inflammatory, antiallergic, prohibiting diabetes complication, preventing coronary heart disease and arrhythmia, antioxidative, scavenging free radicals, antineoplastic and antitumoral activities.

Quercetin has been shown in a number of studies to be potent antioxidants, capable of scavenging free radicals. Quercetin and its derivatives are hydrophilic and lipophilic. Their main antioxidant active groups are phenolic hydroxyl groups. Quercetin can inhibit lipid peroxidation in the body of hypercholesterin model mouse and decrease the blood fat and cholesterin. Quercetin can also inhibit pericellular membrane lipid peroxidation course in human body, enhance the ability of the body's antioxidant defense systems and protect cells from the damage of peroxidation [1,2]. Quercetin and its derivatives are verified as the scavenger of superoxide anion free radicals. The mechanism of their scavenging free radicals and inhibiting lipid peroxidation is chelating transition metal ions to form inactive ion compounds, inhibiting biochemical reactions of ions in the formation of many free radicals, in which the iron ion compounds still maintain the free radical scavenger activity [3,4]. There are three periods in their inhibiting the formation of free radicals in body: inhibiting the induce of free radical reaction by

means of the reaction with superoxide anion ions(O_2^-), inhibiting the formation of the hydroxyl radicals (OH^-) by means of chelating transition metal ions, inhibiting the lipid peroxidation process by means of reacting with lipid peroxidation radicals (ROO^-)[5].

Quercetin, 3, 5, 7, 3′, 4′-pentahydroxylflavone, is a typical flavonol-type flavonoid. Its chemical formula is $C_{15}H_{10}O_7$, its molecular weight is 302.24 and its chemical structure is shown in Fig. 1.

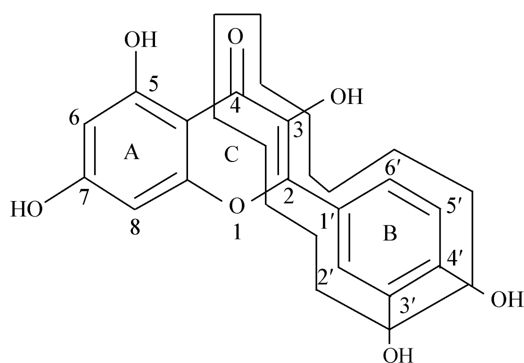

Fig. 1. The chemical structure of quercetin.

Quercetin can also inhibit the oxidative stress damage of neural cells, and has demonstrated some degree of neuroprotection. In this work, we observed the effect of quercetin on the learning and memory behavior of *D*-galactose-induced aged mice in MWM and studied the possible effect of quercetin as an antioxidant on lengthening the aging process of the neural system.

1 Materials and methods

1.1 Chemicals and animals

Quercetin and *D*-galactose were obtained from Sigma, EDTA, mannitol and sucrose from Shanghai First Reagent Factory, and the respective kits of GSH and OH^- from Nanjing Jiancheng Bioengineering Institute, Nanjing, China. All other chemicals were purchased from standard suppliers, and were of the highest purity commercially available. Chemicals were prepared fresh.

Four groups (10 mice in each group) of male mice (20 g±1.0g), which were from the Animal Center of the Nanjing University of Traditional Chinese Medicine, Nanjing, China, were housed under controlled environmental conditions of temperature (22±1℃), light (12 h light/dark) and fed ad lib food and water. All mice were injected for 8 weeks. After the last week, the animals were tested with a group of young male mice (20g±1.0g).

The four groups of mice were injected with *D*-galactose (Sigma) 50 mg/kg. At the same time, the Dgal group was injected with physiologic saline. The other three groups were fed with quercetin (Sigma), 50 mg/kg, 75 mg/kg, and 100 mg/kg.

Eight weeks later, behavior experiments and biochemical tests were conducted for these five groups of mice respectively.

1.2 Morris water maze (*MWM*)

The MWM was 120 cm in diameter and 50 cm in height. The water level in the tank was 1.0 cm above the height of the escape platform, which was 15 cm in height and 6 cm in diameter (Fig. 2). We used a black platform in the pool with the sides and floor painted black to obviate the need for addition of agents to render the water opaque. The animals were tested in the pool for 6 days. Each day, every mouse was placed in the water facing the pool wall in one of the four quadrants (designated NE, NW, SE and SW), allowed to swim (maximum swimming time 90s) until it located the hidden platform. Swimming paths were analyzed by a computer system with a video camera mounted overhead. Four starting positions were used pseudorandomly, and each mouse was trained with four trials per day. After reaching the platform, the mouse was allowed to remain on it for 30 s. If the mouse did not find the platform within 60 s for the first day, the trial was terminated and the animal was put on the platform for 30 s[6,7]. On day 7, the platform was removed from the pool, and the animals were subjected to a 90-s spatial probe trial. We recorded the percentage of swimming time in the quadrant in which the platform had been placed during training over the total swimming time.

1.3 Preparation of homogenates

Following the MWM test, mice were decapitated. The brain was excised. Cerebral cortex and hippocampus were dissected, immediately cooled in ice and washed clean by means of physiologic saline under 4℃. Then, they were put into the glass homogenizer

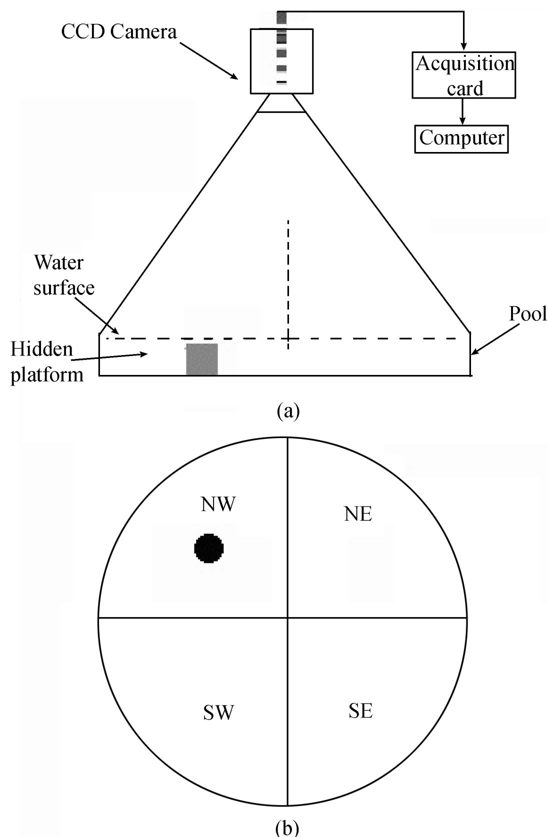

Fig. 2. Diagram illustrating the Morris water maze. (a) The cross section of the system; (b) the top view of the system.

which had the cold physiologic saline water 9 times of the brain tissue weight. They were homogenized in the ice and stored at −80℃ until use.

1.4 The GSH level in brain tissue

The GSH is a low molecular free radicals scavenger and can remove O_2^-, H_2O_2 and so on. The GSH is a small molecule peptide composed of methionine, glycine and cysteine, a main nonprotein sulfhydryl compound in the tissue and a substrate of two enzymes, GPX and GST, and essential to decomposing hydroperoxides of the two enzymes. The GSH can also stabilize sulfhydryl-containing enzymes and protect haemoglobin and other co-factor from the damage of oxidation. When there is GSH, the glutathione peroxidase (GSH-Px) can scavenge superoxide such as H_2O_2 and consume the GSH. The 2-dinitrobenzoic acid (DTNB) was deacidized to TNB-SH_2 by the GSH and formed a yellow compound. 1 mL of brain homogenate (the content of Cys being less than 20 mmol/L, the concentration of GSH less than 0.9 g/L) 0.25 mol/L pH8.0 Tris-HCL buffer and 1 mL 3% formaldehyde were mixed and homogenized. Being put at the room temperature for 60 min, 1 mL of the mixture was added to 5 mL of DTNB analysis solution in the 25℃ water bath and mixed and homogenized. After 5 min, the color developed was read at 412 nm[8]. GSH level was measured according to method of Bradford (1976) and its content was expressed in mg GSH per mg protein.

1.5 The hydroxyl content in brain tissue

The general antioxidation and the ability of scavenging ROS of the antioxidant were measured by means of colorimetry. Being produced in the Fenton reaction, the quantity of OH^- was proportional to that of H_2O_2. In Gress reagent color test, its color developed was red and proportional to the quantity of OH^-. We can measure the ability of scavenging ROS by means of this principle. In the experiment, the operating liquid was prepared according to requirements of the general antioxidation test reagent box (Nanjing Jiancheng Bioengineering Institute). 0.1 mL of brain homogenate and 2.0 mL test reagent were mixed and homogenized, and reacted in the constant temperature water-bath box for 30 min at 37℃. The color developed was read at 520 nm. One Antioxidation Unit standed for every increase of 0.01 of the absorbency value(OD) of the reaction system per milliliter of sample liquid per minute at 37℃. The computing formula is as follows,

The general antioxidation=[(determination OD–Comparison OD)×reaction fluid (mL)×The multiple of dilution before sample test)]÷(0.01×30×sampling quantity (mL))

1.6 *Statistical analysis*

All of the data were analyzed by the one-way analysis of variance (ANOVA).

2 Results and analysis

2.1 *Effect of quercetin on mice induced by D-galactose in MWM*

MWM test was primarily designed to measure spatial learning and memory of mice. Fig. 3 (a) shows

that the escape latency on the first day of training was longer than that on other days for all groups of mice and it decreased gradually during the 6 days of training except the Dgal group of mice. On the sixth day of training, there was obvious difference of latency between the Dgal group and the quercetin treated groups. Fig. 3 (b) shows that the swimming speed of the Dgal group of mice was much lower than that of the Young group, showing aging behaviour, and indicates that quercetin can significantly increase the swimming speed of the mice. Fig. 3(c) illustrates the swimming distance to reach the platform. On the first day of training, most of the animals could not reach the platform without help and the swimming time was mostly 60 s. The swimming speeds of the Young and high dose quercetin treated groups were relatively fast compared with that of the Dgal group, and their swimming distances were mostly longer than that of the Dgal group. Place preference developed gradually. On the final day of the test, the swimming distances of the Young and high dose quercetin treated groups were shortened and the number of the animals that could reach the platform markedly increased. The number of the Dgal aged and low dose quercetin treated group of mice that can reach the platform had almost no change. On the fifth and sixth days of training (Fig. 4), 70% mice of the Young group could reach the platform, while the percentages were 41.32% and 45.45% for the mice treated at 100 mg/kg and 75 mg/kg quercetin, respectively. Only 8.33% mice from the Dgal group and 18.18% mice treated at 50 mg/kg quercetin could reach the platform respectively.

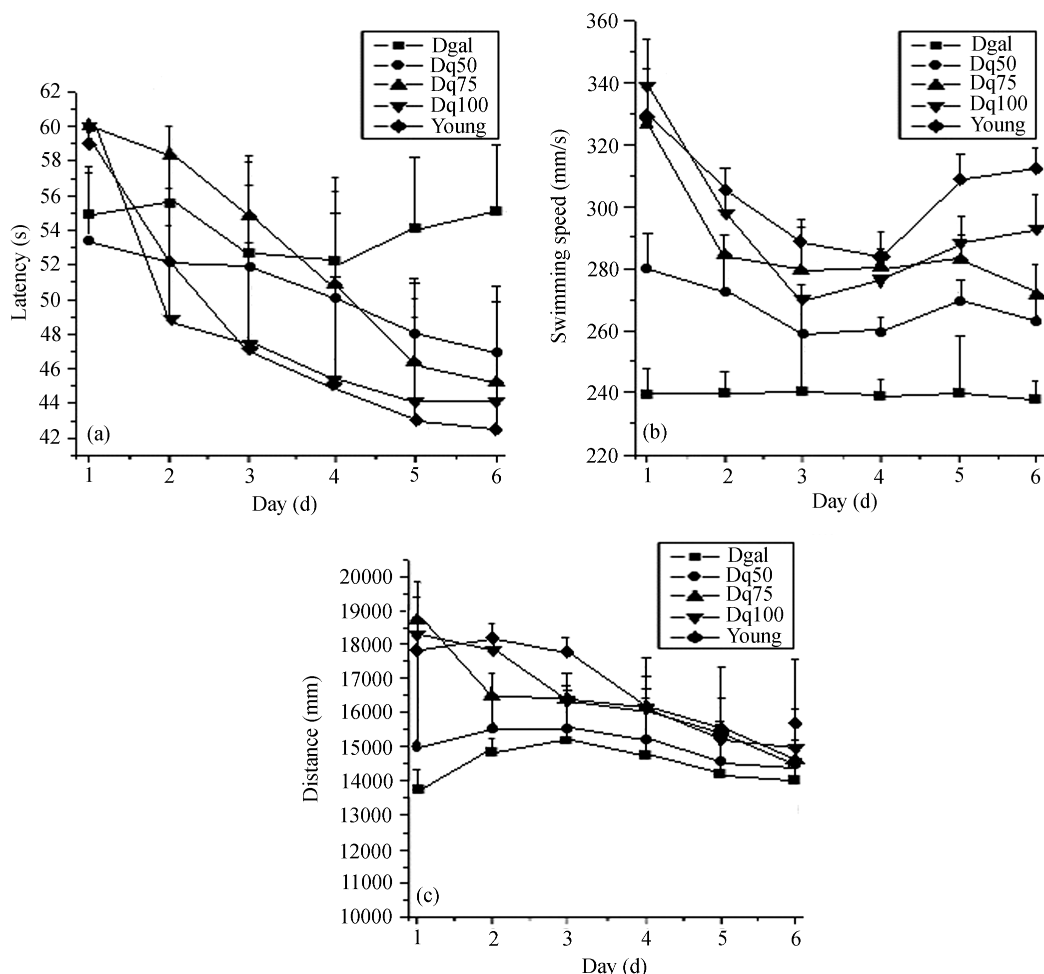

Fig. 3. Effect of quercetin on escape latency (a), swimming speed (b) and swimming distance(c) of the mice with memory impairment induced by *D*-galactose in MWM. Training trials were performed on day 1 to 6 after administration. Young, vehicle-treated group; Dgal, *D*-galactose alone administered 30 min prior to the maze test; Dq50, Dq75 and Dq100, pretreated with the corresponding doses of quercetin + *D*-galactose, quercetin 50, 75 and 100 mg/kg respectively, 30 min prior to the maze test. Data are expressed as means ± S.E.M.

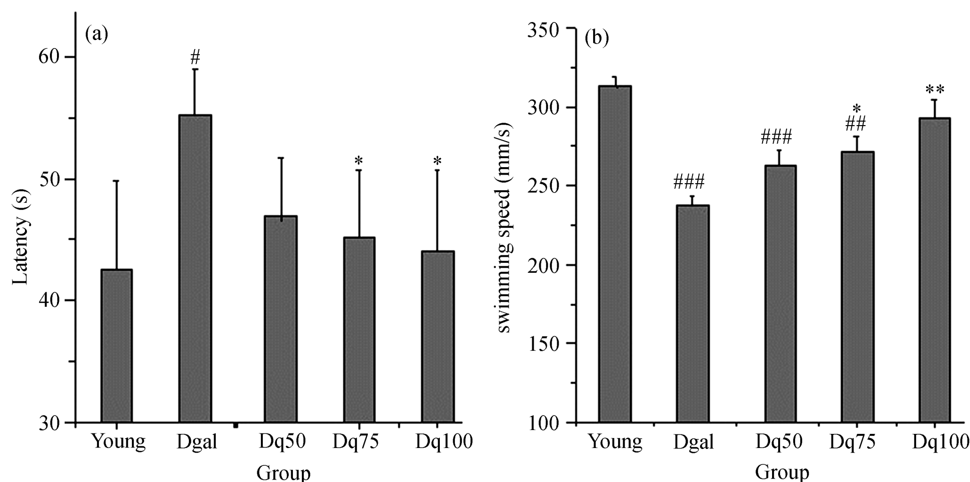

Fig. 4. Effect of quercetin on escape latency (a) and swimming speed (b) of the mice with memory impairment induced by *D*-galactose in the MWM. Training trials were performed on day 1 to 6 after administration. Young, vehicle-treated group; Dgal, *D*-galactose alone administered 30 min prior to the maze test; Dq50, Dq75 and Dq100, pretreated with the corresponding doses of quercetin + *D*-galactose, quercetin 50, 75 and 100 mg/kg respectively, 30 min prior to the maze test. (a) Latency (s) to locate a hidden platform by each group of the mice tested. (b) Swimming speeds averaged across the 6 days of testing. Each bar represents the mean±S.E.M.(N=10 mice per group). No significant differences between groups were found (one-way ANOVA P<0.05). *P<0.05, **P<0.01 vs .D-gal; #P<0.05, ##P<0.01, ###P<0.001 vs. Young group.

After 6 days of training, the escape platform was removed from the water maze and the animals were allowed to swim for 90 s before being removed from the maze. The number of crossings through the previous platform location and the percentage of the total time elapsed in which the mouse was in previous target quadrant were recorded. The result showed that quercetin could increase the number of crossings through the previous platform location of the *D*-galactose-induced aged mice and this number was still less than that of the young group mice (Table 1). The increase of the quercetin dose could increase the swimming time in the previous platform quadrant (Fig. 5).

Table 1 Crossings of platform location (n=10)

Group	Young	Dgal	Dq50	Dq75	Dq100
Times	4.50±2.50	0.70±0.26	0.67±0.22	1.00±0.43	1.42±0.36

Values are expressed as means±S.E.M.

2.2 *Effect of quercetin on the GSH in the hippocampus and cerebral cortex of mice induced by D-galactose*

In the normal state, GSH is an important poiser of cells. Poisonous oxide in cell is periodically removed by means of GSH reduction. The oxidized form of GSH is quickly deacidized by the GSH reductases, and GSH is regarded as the best free radicals scavenger in body. When getting elder, all kinds of free radicals increase in body. The creating of many free radicals can be inhibited by increasing the GSH level in body. It was observed that quercetin can promote the antioxidation of the hippocampus and cerebral cortex of mice by measuring the effect of quercetin on GSH level in mice. Fig. 6 shows that the concentration increase of quercetin can obviously increase the GSH level in the brain tissue of mice. When the quercetin concentration was 50 mg/kg, the GSH level was very different from that of the Dgal group. When it reached 100 mg/kg, the GSH level was comparable to that of the Young group.

2.3 *Effect of quercetin on the hydroxyl content in the hippocampus and cerebral cortex of mice induced by D-galactose*

The hydroxyl free radical(OH^-) is the most active oxygen in chemical property. It can almost react on all kinds of organic matter, such as sugar, amino acid, phosphatides, nucleotide, organic acid. Due to its strong reactivity with biomolecules, OH^- is probably capable of doing more damage to biological systems than any other ROS. Hydroxyl measure box was an active oxygen reagent box. It can produce hydroxyl free radicals and the outcome can reveal the strength

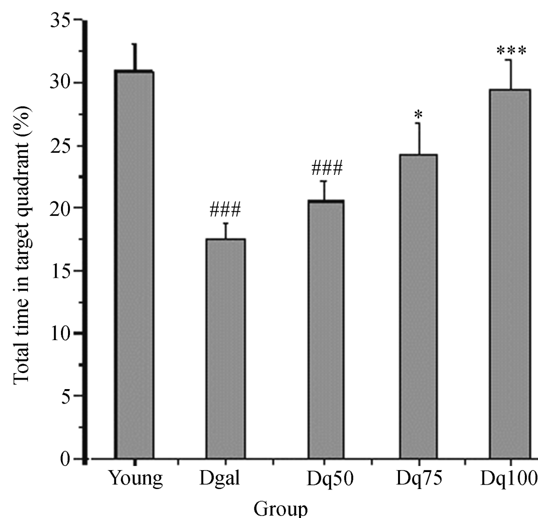

Fig. 5. Effect of quercetin on transfer test in the MWM. Young, vehicle-treated group; Dgal, *D*-galactose alone; Dq50, Dq75 and Dq100, pretreated with the corresponding doses of quercetin + *D*-galactose, quercetin 50, 75 and 100 mg/kg respectively. The % total time spent in seconds in the previous target quadrant is presented. Each bar represents the mean ± S.E.M. of trials per mouse in each group. *N* = 10 mice per group.(One way ANOVA *P*<0.05). **P*<0.05, ****P*<0.001 vs .Dgal; ###*P*<0.001 vs. Young group.

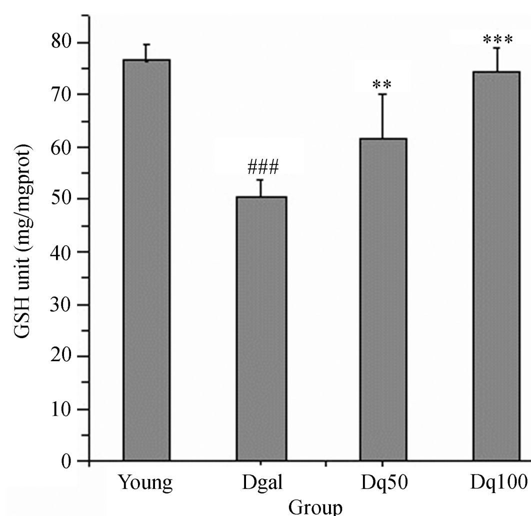

Fig. 6. Effect of quercetin on the GSH in the hippocampus and cerebral cortex of the mice induced by *D*-galactose. The GSH content in the hippocampus and cerebral cortex of different groups of mice. Young, vehicle-treated group; Dgal, *D*-galactose alone; Dq50 and Dq100, pretreated with the corresponding doses of quercetin + *D*-galactose, quercetin 50 and 100 mg/kg respectively. Means ± S.E.M. is represented. ***P*<0.01, ****P*<0.001 vs .Dgal; ###*P*<0.001 vs. Young group.

of antioxidation of tissues. Fig. 7 illustrates that the more the quercetin dosage, the stronger the antioxidation of tissues. When the concentration of quercetin was 100 mg/kg, the antioxidation of the hippocampus and cerebral cortex of the mice was comparable to that of the Young group.

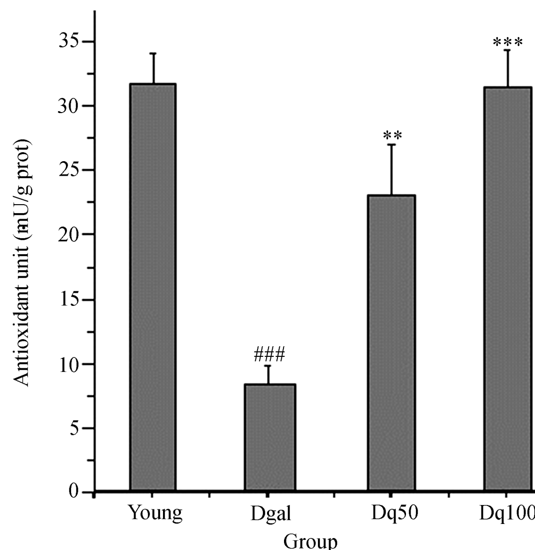

Fig. 7. Effect of quercetin on the hydroxyl content in the hippocampus and cerebral cortex of the mice induced by *D*-galactose. The antioxidant unit in the hippocampus and cerebral cortex of different groups of mice. Young, vehicle-treated group; Dgal, *D*-galactose alone; Dq50 and Dq100, pretreated with the corresponding doses of quercetin + *D*-galactose, quercetin 50 and 100 mg/kg respectively. Means ± S.E.M. is presented. ***P*<0.01, ****P*<0.001 vs .Dgal; ###*P*<0.001 vs. Young group.

3 Discussion

Aging is a natural and inevitable part of the life process that is characterized by a gradual and general decline in physiological and motor functions. The “free radical hypothesis of aging” claims that, along with aging, the generation and accumulation of ROS (such as superoxide and hydroxyl radicals) result in oxidative damage to the critical biological molecules, which, coupled with the insufficiency of endogenuous antioxidant defense mechanisms, contributes to the detrimental effects of aging[9]. The free-radicals defense systems of body, including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) should keep the oxidative processes in check and protect the body from the damage[10]. SOD and GSH-Px were the main antioxidase of scavenging free radicals of body. Their content indirectly reflected the ability of scavenging free radicals. Malondialdehyde(MDA) levels, an indicator of lipid peroxidation, were increased several times with age in cells from the

mice. MDA was one of the products of the lipid peroxidation induced by free radicals in body. As getting old, the function of the free-radicals defense system of body decreased, the SOD in body gradually decreased, and MDA gradually increased. This induced a lot of unsaturated fatty acid to oxidize and damage biomembrane, DNA, protein and so on, indicating the relation between the aging of body and free radicals.

In this experiment, the *D*-galactose-induced aged mice model was used. The mice were injected with the *D*-galactose in a continuous period of time, and the concentration of the galactose in body cells increased. Under the catalysis of aldose reductases, the *D*-galactose deacidized to galactitol. Galactitol could not be further metabolized, and thus accumulated in the cells, influencing the normal osmotic pressure and leading to cell swelling, function obstructing, metabolic disorder, damaged and consummed antioxidant defense system of body and accumulated free radicals. During the deacidizing course of galactose, ROS was created, the pericellular membrane was damaged, and peroxide lipid was increased, all of which speeded up the aging process.

Quercetin was an effective free radical scavenger and antioxidant. Experiments showed that flavonoid compounds had good scavenge effect on removing superoxide anion ions (O^{2-}), hydroxyl free radicals (OH^-) and singlet ($^1O_2$). This effect may relate with 3, 7-hydroxyl group on its chemical structure[11]. Further studies showed that quercetin can combine with Cu^{2+},Fe^{1+} and Mn^{2-}. Its antioxidation was possibly realized by affecting iron ion equilibrium within body and changing the oxidation status in cell. Quercetin can combine superoxide ion to decrease the production of hydroxyl radicals, complex iron ions to inhibit the formation of hydroxyl radicals and react with ROO^- to prohibit the lipid peroxidation process. Quercetin can inhibit aldose reductases to decrease the consumption of NADPH and to enhance the antioxidation of body[12]. A lot of work on antioxidation of quercetin had been reported, but seldom dealt with the central nervous system. Only a few cases dealt with the function of nerve cells cultured out of the body. This experiment showed that quercetin could improve the learning and memory capability of the aging model animals. The hippocampus and cerebral cortex of the mice was considered the most important brain areas related with the learning and memory[13]. Up to now, there has been no report concerning whether quercetin has inhibition effect on the oxidation damage to these two brain areas during the aging process.

Aging is accompanied with the declining capability of spatial learning and memory, which is coordinated with different brain regions. Since the oxidative damage may play a certain role in the aging process, including the associated cognitive decline, age-related impairment in spatial learning and memory may be alleviated by antioxidant treatment. The experiment showed that, in the hippocampus and cerebral cortex of mice fed with quercetin, the content of hydroxyl radicals decreased, while that of GSH increased. GSH can scavenge free radicals, so the increase of its content can improve the GSH-Px activity. The main biological function of GSH-Px is scavenging lipid peroxide and substituting CAT to scavenge H_2O_2 in the tissue where the content of CAT is extremely low. As one of the enzymes scavenging ROS, GSH-Px can prevent aberration and can be involved in the PGs synthesis. It also has other important pathological and physiological functions. For example, it can improve the endogenous antioxidant, protect free-radicals defense mechanism and prevent aging[14]. All this shows that quercetin can inhibit the decrease of GSH in the hippocampus and cerebral cortex of mice induced by *D*-galactose and improve the cognitive function of the aging model mice.

In conclusion, quercetin exhibits a potent cognition-improving function in memory-impaired mice subjected to *D*-galactose insult. The underlying mechanism of this action may predominantly involve the antioxidant effects of quercetin, increase of the GSH level and decrease of the hydroxyl radicals content in the hippocampus and cerebral cortex of mice. All these factors may lay good foundations for making full use of quercetin as a natural anti-aging pharmaceutical product.

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