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Oviductus Ranae Improves Cognitive Disorder and Suppresses Oxidative Stress in Aging Mice by Activating Nrf2 Pathway

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ABSTRACT

Oviductus ranae (OR), the dried oviduct of mature female Rana dybowskii, also known as Hamayou and Hashimayou, is a famous animal derived traditional Chinese medicine that have a variety of pharmacological effects. In our study, the anti-aging effect of OR on 53-week-old aging mice was investigated. The results showed that OR could significantly shorten the destination quadrant distance and latency in the Morris water maze of aging mice. It obviously decreased the MDA, MAO-B and Lipo level in brain tissue, enhanced the SOD and GSH-Px activity in brain tissue, and increased the IgA, IgG, IL-1 and IL-2 level in serum. In addition, OR significantly improved the protein expressions of Nrf2, HO-1 and NOQ1 in the brain tissues of aging mice, which was detected by western blotting. In conclusion, OR treatment improved behavioral disorders and brain damage in the aging mice, suggesting that OR has the potential to be a new anti-aging drug candidate.

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Key words
Oviductus ranae, Aging mice, Nrf2 pathway, Oxidative damage, Anti-aging drug, MDA, MAO-B and Lipo, IgA, IgG, IL-1 and IL-2

INTRODUCTION

Aging is an inevitable phenomenon, which occurs in all living things including animals and humans. It is the degeneration of the body and organs, the main manifestations are the gradual decline of brain function and the progressive loss of memory (Campisi *et al*., 2019; Flatt, 2012). With the increasing proportion of the elderly population, it is urgent to study the aging mechanism and effective anti-aging medicines (Cebe *et al*., 2014). The process of aging involves many complex elements. Whereas, many reports have revealed that free radicals play an important role in the occurrence and development of aging (Sendama, 2020). Studies have confirmed that a series of negative effects caused by intracellular free radicals and ROS can lead to pathological damage of neuronal cells, slow down neuronal cell metabolism, reduce neuronal cell activities, and damage neuronal cells, resulting in the decline of the cognitive function due to aging (Waly *et al*., 2012). Oxidative stress causes an imbalance between the systemic manifestation of reactive free radicals and the repair ability of biological systems, resulting in DNA damage and chromosomal aberration. At present, many long-lived species reduce their own oxidative stress response and maintain their internal oxidative balance through evolution (Hoffman *et al*., 2017; Salmon *et al*., 2009). Therefore, alleviating oxidative stress in vivo may be an effective treatment strategy for anti-aging or prevention of age-related cognitive dysfunction.

Oviductus ranae (OR) is a kind of Chinese medicine extracted from *Rana chensinensis*, which has a wide range of pharmacological activities (Li *et al*., 2019; Wang *et al*., 2013). Pharmacological studies have shown that it has the effects of anti-aging, anti-oxidation, anti-fatigue and reducing blood lipid (Zhang *et al*., 2019; Shen *et al*., 2012). Some studies have also shown that OR can alleviate some of the symptoms of menopause (Zhang *et al*., 2018; Wang *et al*., 2013). In addition, it has been revealed that OR can promote the proliferation of granulosa cells (Ling *et al*., 2017). Nuclear factor erythroid 2-related factor 2 (Nrf2) is a member of the leucine zipper transcription factor family. It is considered to be one of the key regulatory factors of cell protection and detoxification genes, which can reduce the oxidative stress injury (Sharma *et al*., 2020).

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Normally, KEAP1 (Kelch-like ech-associated protein 1) binds to Nrf2 in the cytoplasm. After the oxidative stress, Nrf2 is released and transferred to the nucleus, where it binds to the antioxidant response element (ARE), activates the transcription of downstream genes, and induces a series of antioxidant reactions and reactive phase II detoxification enzymes, including heme oxygenase 1 (HO-1) and NADPH quinone oxidase 1 (NQO1) (Sykiotis and Bohmann, 2010; Surh, 2003). Increasing evidences also show that Nrf2 pathway may be damaged in the process of aging in the body. Compared with normal mice, Nrf2-deficient mice are more prone to oxidative stress response, and overexpression of Nrf2 shows a better neuroprotective effect (Buendia *et al*., 2016; Loboda *et al*., 2016).

To further clarify these mechanisms, in this work, the effects of OR on improving cognitive impairment caused by aging in mice, and its possible mechanisms were investigated. It is speculated that OR may improve memory impairment caused by natural aging, and its mechanism may be related to Nrf2 pathway. The study is expected to provide a reference for the development of anti-aging application of OR.

MATERIALS AND METHODS

Chemicals and reagents

Oviductus ranae (OR), derived from adult female Chinese forest frog (*Rana chensinensis*), was purchased from Jilin Province, China. After freeze-drying, OR was crushed through 80 mesh sieve. During administration, the powder of OR was weighed quantitatively and put into a beaker. Then added distilled water at 70°C and stirred well. The OR powder was fully dissolved and expanded after being placed at room temperature for 12 hours. Then distilled water was added to constant volume to prepare the desired concentration. The antibodies against Nrf2, HO-1 and NQO1 were purchased from Santa Cruz Biotechnology Inc. (Dallas, TEX, USA). ELISA kits (for IgA, IgG, IgM, IL-1, and IL-2) and biochemical kits of MAO-B, Lipo, SOD, MDA and GSH-Px were purchased from Nanjing Jiancheng Bioengineering Inc. (Nanjing, China).

Animal model

The 53 week old female ICR mice (set as aging mice, n=40) and 6 weeks old female ICR mice (set as young mice control group, n=10) were obtained from Liaoning Changsheng Biotechnology Co. Ltd. The mice were placed in a cage (6 mice per cage) in a controlled environment (23-25°C, humidity 60-70%), and animals were provided with sufficient food and drinking water to eat and drink freely in a natural circadian rhythm.

Morris water maze test

Morris water maze (MWM) test was carried out 22 days after administration. A certain amount of water was injected into the MWM device (diameter 120cm, height 50cm). The water temperature was controlled at 24±2°C and the depth was 23cm. Selected 4 entry points, and divided the maze into 4 quadrants. A transparent plastic platform (10 cm in diameter and 22 cm in height) was placed in the first quadrant of 2cm underwater and remained in place during the experiment. Skimmed milk powder was added to the water to prevent the mice from seeing the platform. The mice were put into the water successively from the water entry points in each quadrant facing the pool wall, and were allowed to stay on the platform for 20 s after climbing up to the platform. The next test was conducted after an interval of 60 s. If the mouse failed to find the platform within 60 s, it would be led to the platform and stay for 20 s. After 5 days of continuous training, the platform was taken out 1h after administration on the 6th day. The mice were put into the water at the entry point of the relative quadrant in the quadrant where the original platform was located, and the distance through the target quadrant where the original platform was located and the time required to reach the platform and stand up after entering the water (as escape latency) were recorded within 60 s.

Oxidative stress level test

After the MWM experiment, the mice were sacrificed, and the brain tissues were quickly extracted from ice to prepare 10% tissue homogenous serous fluid. The supernatant was frozen for later use, and the activities or contents of MAO-B, SOD, GSH-Px, LiPO and MDA were measured.

Immune factors level test

Blood samples were taken from mice and centrifuged at 3500 r/min for 10 min. Serum samples were separated and stored at -80°C. The contents of IgA, IgG, IgM, IL-1 and IL-2 in serum were determined by ELISA.

Western blot analysis

The Western blot was used to detect the expression levels of Nrf2, HO-1 and NQO1 proteins in mouse brain tissue. The brain tissue proteins were extracted with protein extraction reagent, harvested and frozen in the RIPA buffer for 30min. Protein concentration was determined by BCA protein detection kit. Brain tissue protein (20 μL) was loaded on 12% polyacrylamide-SDS gel. After electrophoresis, the gel was coated on PVDF membrane and sealed with 5% (w/v) skim milk for 1h. Then incubated the transfer membrane with the appropriate primary antibody overnight at 4°C. The enzyme-labeled secondary

antibody were used to detect the binding of the primary antibody, and the ECL chemiluminescence was visualized.

Statistical analysis

The SPSS 26.0 was used for data analysis. All the experimental data were expressed as mean±SD. Differences between groups were compared by one-way analysis of variance (ANOVA). When $P<0.05$, the difference is considered to be statistically significant.

RESULTS

Cognitive impairments

Figure 1 shows the effect of OR on cognitive impairments in aging mice. According to the results of MWM test, it took significantly longer for aging mice to reach the platform than young mice, and the time required for young mice to reach the platform was shortened by the hiding platform experiment training (Fig. 1A). In addition, the aging mice that were not given the drug showed a significantly higher latency in the test, suggesting that the mice had a learning deficit. After OR treatment, the escape latency was obviously shorter than untreated aging mice. Compared with young mice, the total distance in the target quadrant of aging mice was significantly increased, while the distance in the OR treatment group was significantly shortened (Fig. 1B).

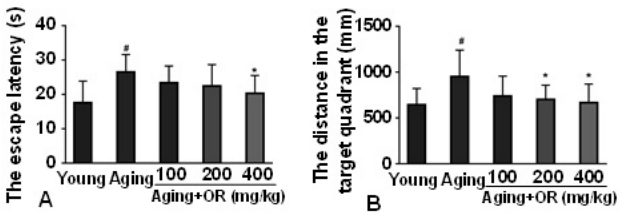

Fig. 1. The effect of OR on the cognitive impairments in aging mice. (A) the escape latency and (B) the distance in the target. Notes: Compared with the young mice group, #$P<0.05$; compared with the aging mice group, *$P<0.05$.

Oxidative stress

Figure 2 shows the effect of OR on oxidative stress in the aging mice. The activities of SOD and GSH-PX were obviously increased in the group of OR, (Fig. 2A, 2B) while the contents of MDA and Lipo were obviously decreased compared with the untreated aging group (Fig. 2C, 2E). And the content of MAO-B showed a decreasing trend, but there was no statistical difference (Fig. 2D). The results showed that the aging mice showed oxidative stress injury. However, treatment with OR may ameliorate these changes.

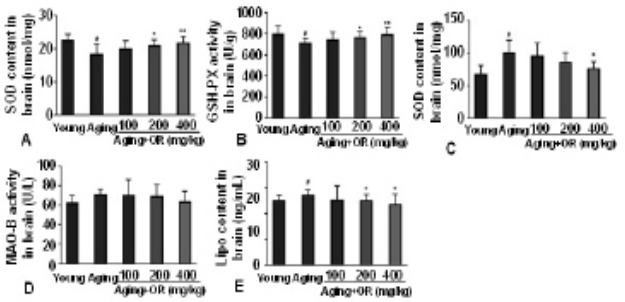

Fig. 2. The effect of OR on oxidative stress in the hippocampus of aging mice. Activities of brain (A) SOD and (B) GSH-PX, (C) content of brain MDA, (D) activitys of brain MAO-B, (E) content of brain Lipo. Compared with the young mice group, #$P<0.05$; compared with the aging mice group, *$P<0.05$, **$P<0.01$.

Immune factors

Figure 3 shows the effect of OR on immune factors of aging mice. Compared with untreated aging group, the contents of IgA and IgG in serum of OR group were significantly increased (Fig. 3A, 3B), and the levels of IL-1 and IL-2 in serum were significantly increased (Fig. 3D, 3E). And the content of IgM showed no obvious trend of change (Fig. 3C). These results suggested that OR could regulate the immune function of aging mice.

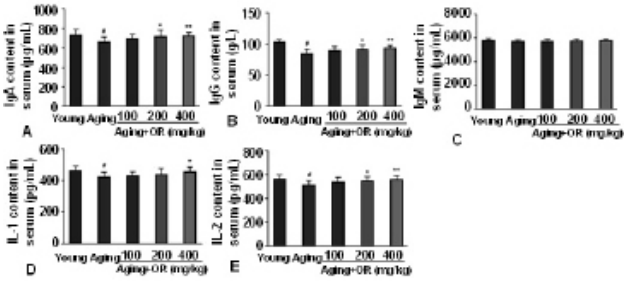

Fig. 3. The effect of OR on immune factors of aging mice. Content of serum (A) IgA, (B) IgG, (C) IgM, (D) IL-1 and (E) IL-2. Compared with the young mice group, #$P<0.05$; compared with the aging mice group, *$P<0.05$, **$P<0.01$.

Nrf2 pathway

Figure 4 shows the effect of OR on the Nrf2 pathway in aging mice. In order to investigate the regulatory effect of OR on Nrf2 pathway, we detected the expression of Nrf2, HO-1 and NQO1 proteins in brain tissues. It can be seen from Figure 4, compared with untreated aging group, the expression of Nrf2, HO-1 and NQO1 was clearly decreased, while OR reversed the decline of Nrf2, HO-1 and NQO1 expression in brain tissue (Fig. 4 A-D).

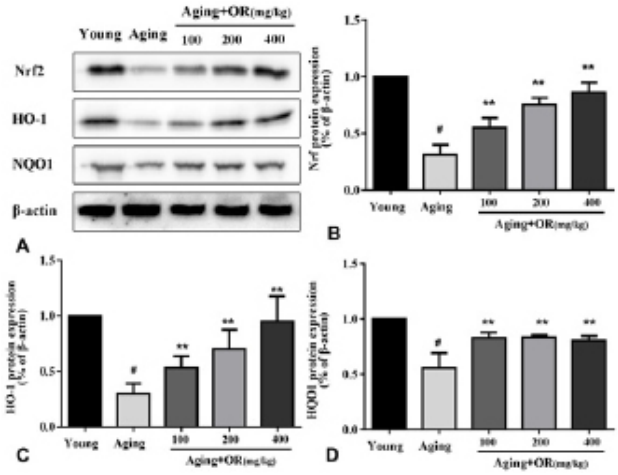

Fig. 4. The effect of OR on the Nrf2 pathway in aging mice. Nrf2, HO-1 and NQO1 expression were analyzed by western blot (A), Quantification graphs of Nrf2, HO-1 and NQO1 are shown on (B), (C) and (D). Compared with the young mice group, #P<0.05; compared with the aging mice group, **P<0.01.

DISCUSSION

In this research, the biological function of OR has been well evaluated, and its antioxidant capacity improves working memory damage caused by aging. The mechanism of action may be related to the reduction of oxidative stress damage, which is similar to the results of SOD/MDA measurements in brain tissue and blood. We also found that the levels of inflammatory cytokines were inhibited during OR treatment, suggesting that OR exhibits the neuroprotective function in the context of brain inflammation. In addition, the activation of Nrf2 participates in the beneficial effects of OR, which is also considered to be a potential mechanism of its function. Memory impairment is a typical first symptom of neurodegenerative diseases. The patient's learning and memory abilities are impaired, and short-term memory is impaired. Some research indicated that the learning ability of passive avoidance in 2 months old mice is impaired, and its learning and memory impairment (MWM) can detect various behavioral tasks in 4-6 months old mice (Yi *et al*., 2016; Zhang *et al*., 2013). And many similar studies have shown that aging mice exhibit age-related cognitive decline, which is consistent with human cognitive decline (Hou *et al*., 2019). From the results of this study, 53 weeks old mice developed memory impairments during the MWM test. The administration of oral OR significantly improved this impairment. Therefore, OR may improve the cognitive function of aging mice by preventing brain learning and memory impairment.

Oxidative stress is one of the most common hypotheses leading to aging (Wu *et al*., 2015). Oxidative stress causes cognitive dysfunction by affecting synaptic plasticity and neuronal activity (Hybertson *et al*., 2011). In aging mice, the production level of oxidants from various sources increased, while the expression level of antioxidant enzymes, which is an important line of defense, decreased (Chen and Zhong, 2014). During the aging process, the activity of the repair system is also inhibited to cope with the decline in metabolic capacity. In our research, we found that the MDA of aging mice increased and the activity of SOD and GSH-Px decreased. OR treatment could significantly reverse these changes. MDA is the product of the peroxidation reaction of polyunsaturated fatty acids and is widely regarded as a biomarker of free radical-mediated lipid peroxidation damage. SOD and GSH-Px are important components in the antioxidant defense system, which are mainly responsible for the conversion of superoxide (O_2) to hydrogen peroxide (H_2O_2) (Tönnies and Trushina, 2017).

Nrf2 is an important factor of oxidative stress, and regulates the expression of antioxidant proteins by combining with ARE. Under normal physiological conditions, Nrf2 is located in the cytoplasm, binds to Keap1, and is rapidly degraded by the ubiquitin-proteasome pathway (Bellezza *et al*., 2018; Uruno and Motohashi, 2011). When exposed to electrophiles or oxidative stress, Nrf2 separates from Keap1 and transfers to the nucleus. Then, Nrf2 that enters the nucleus binds to ARE, promotes the transcription of downstream target genes HO-1 and NQO1, and jointly inhibits oxidative stress. Hence, the expression of downstream target genes plays a key role in maintaining redox homeostasis (Zhu *et al*., 2016; Yao *et al*., 2014; Kwak *et al*., 2002). We assume that OR may activate Nrf2 by inhibiting the protein Keap1, thereby dissociating the Keap1-Nrf2 complex. In our research, we found that OR treatment activated the Nrf2 signaling pathway in aging mice, thereby having enhanced the expression of downstream antioxidant proteins, suggesting that the antagonistic effect of OR on aging mice is related to Nrf2.

CONCLUSIONS

In summary, this work indicated that OR played an anti-aging role by improving brain function, anti-oxidative stress and enhancing immunity. The mechanism of OR may be delay brain oxidative stress damage in aging mice by regulating the Nrf2 pathway. It is expected to become an ideal target for OR to prevent aging-related diseases in the future.

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Ethical compliance

All experiments in this paper were conducted in accordance with the guidelines for animal research, and were certified by the Ethics Committee of Animal Experiments of Changchun University of Chinese Medicine (Certificate No.: 2019021).

Statement of conflicts of interest

The authors have declared no conflict interest.

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