Anthocyanidin Extract from Summer-black-grape Affects the Expression of Ki-67 in Testis, Ovary of D-Galactose-induced Aging Mice

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Abstract: The aim of this work was to analyze the expression of Ki-67 in ovary, testis of aging mice with anthocyanidin extract from Summer-black-grape. ICR mice (the aging group and the anthocyanidin-groups (50 mg/kg/D-group, 75 mg/kg/D-group and 100 mg/kg/D-group) were employed to evaluate the effect of grape anthocyanidin on reproductive system. The results showed that the anthocyanidin had strong scavenging ability for free radicals, the level of oxidation in serum of mice treated with anthocyanidin was low, and the pathological changes were not obvious. In the anthocyanidin group, the Ki-67 positive particles in the testis and ovary tissue were significantly decreased. The anthocyanidin of Summer-black-grape reduces the expression of Ki-67 protein in the testis or ovary of aging mice. In gonadal cells of aging mice, the anthocyanidin were shown protective effect in the proliferation.

Key words: summer-black-grape, anthocyanidin, anti-aging, aging model, Ki-67, reproductive system

1 Introduction

Anthocyanidin are polyphenolic pigments that belong to the flavonoid group and are responsible for many of the red-orange to blue-violet colors present in plant organs such as fruits, flowers, and leaves. Anthocyanidin-rich foods such as bilberries and grapes have traditionally been recommended in Europe and Asia for treatment of atherosclerosis, chronic venous insufficiency, and other health effects. Triploid seedless grape, nicknamed Summer-black-grape, is a hybrid of Europe and America. Summer-black-grape has thick skin and black purple color, its flesh is crisp and purple red. It tastes sweet and has excellent quality. Anthocyanidin are mainly extracted from grape skins and grape seeds. They have been found to have anti-aging, anti-inflammatory and enhanced learning and memory functions.

Aging is a complex and inevitable biological process that is associated with numerous chronically debilitating health effects, such as coronary heart disease, diabetes, Alzheimer's disease. Development of effective interventions for promoting healthy aging is an active but challenging area of research. Galactose (D-gal) is thought to be used to make artificial aging models. As a marker of cell proliferation activity, the expression of Ki-67 was immunohistochemically evaluated. Herein, we studied the effects of anthocyanidin on cell proliferation by monitoring the expression of Ki-67 in ovary, testis of aging mice.

2 Materials and Methods

2.1 Laboratory animals pharmaceutical reagents

ICR mice (2 month old) were purchased from Beijing Weitong Lihua Experimental Animal Technology Co., Ltd. (Beijing, China), animal certificate number is SCXK (Beijing)2016-0006. The mice housed in specific pathogen free conditions at a temperature of 20-25°C, a 50-70% humidity, a light/dark cycle of 12/12 h, and with free access to

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water and food. All procedures complied with the guiding principles for the care and use of animals and were approved by the Committee on Ethics of Animal Experimentation at Zhengzhou University (Zhengzhou, China).

2.2 Pharmaceutical reagents
Summer-black-grape anthocyanidin was extracted and supplied by the School of Materials, Zhengzhou University of this project. The other reagents are pure domestic analysis.

2.3 Main instrument
Main instrument contains 755S ultraviolet spectrophotometer (Shanghai Lingguang Technology Co., Ltd. Shanghai, China); PICO17 centrifuge, Thermo Company (USA); BH-2 binocular microscope, Olympus Company (Japan); GD100-S18 water bath, Grant Company (UK).

2.4 Experimental grouping and control group processing
The 40 mice received hypodermic injection of D-galactose (300 mg/kg/D) for 55 days to construct the mouse aging model. 40 mice were divided into four groups at random (aging group, 50 mg/kg/D-group, 75 mg/kg/D-group and 100 mg/kg/D-group). In the aging group, saline was given in the same volume intragastrically. In the 50 mg/kg/D-group, Summer-black-grape anthocyanidin (50 mg/kg/day) was given intragastrically into the mice daily for 30 days. In the 75 mg/kg/D-group, Summer-black-grape anthocyanidin (75 mg/kg/day) was given intragastrically into the mice daily for 30 days. In the 100 mg/kg/D-group, Summer-black-grape anthocyanidin (100 mg/kg/day) was given intragastrically into the mice daily for 30 days. The mice housed in specific pathogen free conditions at a temperature of 20-25℃, a 50-70% humidity, a light/dark cycle of 12/12 h, and with free access to water and food. In the process of modeling, each group of mice was observed for mental state, coat color, activity and water intake, and the weight was measured daily.

2.5 Specimen collection and index determination
On the 31st day, the mice were sacrificed by cervical dislocation. We collect blood through the eyelids of mice. The body weight evolution of the three anthocyanidin groups and aging group mice can be appreciated in Table 1 and Fig. 1. The weights in aging group, 50 mg/kg/D-group, 75 mg/kg/D-group and 100 mg/kg/D-group were 25.2300 ± 1.0112g, 24.7537 ± 1.1450g, 24.6460 ± 0.8532g, 24.6320 ± 1.3122g, respectively. The comparisons between each group and 100 mg/kg/D-group were 25.2300 ± 1.0112 compared to the aging group.

| Groups               | n  | Body weight (g)   |
|----------------------|----|-------------------|
| Aging group          | 10 | 25.2300 ± 1.0112  |
| 50 mg/kg/D-group     | 10 | 24.7537 ± 1.1450  |
| 75 mg/kg/D-group     | 10 | 24.6460 ± 0.8532  |
| 100 mg/kg/D-group    | 10 | 24.6320 ± 1.3122  |

(a: p > 0.05 compared to the aging group)
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The results showed that weight of three anthocyanidin groups were not statistically significant compared with aging group.

3.2 Morphological changes of the D-gal-induced aging testis and ovarian tissues

There were no significant differences in the organ morphology among the four groups. Microscopic examination revealed that the structure and arrangement of the seminiferous tubules of the male mice were disordered in aging group. Compared with the aging group, the sperm epithelium is intact and the spermatozoa are regularly arranged in the anthocyanidin groups. We also observed that the number of spermatozoa/support cells decreased in the aging group, and the number of cells increased after anthocyanidin treatment (Fig. 2). In the aging group, D-gal caused damage to granulosa cells in the ovarian tissue. The granulosa cells were loosely and irregularly arranged, and the follicles also changed. These adverse changes in growing follicles and granulosa cells were alleviated by anthocyanidin treatment (Fig. 3). These data indicate that anthocyanidin can reverse D-gal-induced damage to follicular structure growth.

3.3 Anthocyanidin affects oxidation-associated biomarkers in aged mice

The content of SOD in aging group, 50 mg/kg/D-group, 75 mg/kg/D-group and 100 mg/kg/D-group were 181.024 ± 2.146 μg/mg, 188.352 ± 9.612 μg/mg, 191.008 ± 10.903 μg/mg, 203.872 ± 7.189 μg/mg, respectively (Table 2). The comparisons between each group had significant differences (p ≤ 0.05). The content of MDA in aging group, 50 mg/kg/D-group, 75 mg/kg/D-group and 100 mg/kg/D-group were 13.518 ± 2.043 nmol/mg, 9.821 ± 3.083 nmol/mg, 5.981 ± 3.227 nmol/mg, 3.050 ± 2.580 nmol/mg, respectively (Table 2). The comparisons between each group had significant differences (p ≤ 0.05).
In the present study (Table 2 and Fig. 4), SOD activity was increased significantly, and MDA content was decreased significantly, in the anthocyaninidin groups compared with the control. In the 100 mg/kg/D-group, SOD activity was significantly increased compared with the other group. In addition, MDA content was decreased in the 100
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Table 3  The positive expression rates of Ki-67 in serum of mice in each group (mean ± standard deviation, n = 10).

| GROUPS      | n  | The positive expression rates of Ki-67 in testis | The positive expression rates of Ki-67 in ovary |
|-------------|----|-------------------------------------------------|---------------------------------------------|
| Aging group | 10 | 24.70% ± 4.33%                                   | 63.30% ± 9.03%                              |
| 50 mg/kg/D-group | 10 | 19.52% ± 2.87%                                   | 51.27% ± 7.23%                              |
| 75 mg/kg/D-group | 10 | 11.55% ± 1.83%ab                                  | 28.12% ± 3.54%ab                            |
| 100 mg/kg/D-group | 10 | 7.21% ± 1.33%ab                                  | 25.07% ± 3.76%ab                            |

(a: p<0.05 compared to the aging group; b: p<0.05 compared to the 50 mg/kg/D group; c: p<0.05 compared to the 100 mg/kg/D group)

Table 3 shows the positive expression rates of Ki-67 in serum of mice in each group. The expression of Ki-67 decreased with the increase of anthocyanin concentration.

Fig. 5  The positive expression rates of Ki-67 in serum of mice in each group (n = 10/group). The expression of Ki-67 decreased with the increase of anthocyanin concentration.

3.4 Affect Ki-67 expression in the testis, ovary of aging mice

The positive expression rates of Ki-67 in testis in aging group, 50 mg/kg/D-, 75 mg/kg/D-group and 100 mg/kg/D-group were 24.70% ± 4.33%, 19.52% ± 2.87%, 11.55% ± 1.83%, 7.21% ± 1.33%, respectively (Table 3 and Fig. 5). The comparisons between each group had significant differences (p ≤ 0.05). The positive expression rates of Ki-67 in ovary in aging group, 50 mg/kg/D-group, 75 mg/kg/D-group and 100 mg/kg/D-group were 63.30% ± 9.03%, 51.27% ± 7.23%, 28.12% ± 3.54%, 25.07% ± 3.76%, respectively. The comparisons between each group had significant differences (p ≤ 0.05). In the present study, the results demonstrated that the positive expression rates of Ki-67 in testis were decreased significantly in the anthocyanidin group compared with the control. In the 100 mg/kg/D-group, the positive expression rates of Ki-67 were significantly decreased compared with the other group. These results demonstrated that the protective effect of anthocyanidin on the testis, ovary.

4 Discussion

Aging is the universal and gradual process of recession of all organs. The aging of the reproductive system begins first in a person’s life. The aging of the reproductive system affects the function of other organs, declining the function of other systems. It has been reported that the decline of male physiological and sexual functions is associated with aging of the male reproductive system. Therefore, the fight against aging must first delay the aging of the reproductive organs.

The D-galactose-induced aging mice model is based on the oxidative stress theory. The normal dose of D-galactose is a reduced aldose in the body, which can be metabolized to glucose in the liver. Excessive D-galactose body cannot be metabolized. It induces the production of reactive oxygen species, which reduces the level of SOD in the serum of mice, increases the content of MDA, and causes tissues and organs to enter aging. SOD is an important enzyme...
free radical scavenging antioxidant enzyme in the human body. It is present in the cell fluid. It can scavenge excess free radicals, avoiding protein denaturation and lipid peroxidation. SOD levels are reduced with the aging of the body, causing free radicals, so the SOD level can indirectly reflect the body’s antioxidant capacity\textsuperscript{12}. MDA is a lipid peroxidation product in the body. MDA can cross-link with proteins and DNA, and chemically react with phospholipids on the cell membrane to destroy the cell membrane structure and damage the cells\textsuperscript{13}. Therefore, MDA is an important indicator for judging the degree of peroxidation in the body, which can determine the degree of tissue oxidation and the degree of aging of the body\textsuperscript{14}. In this experiment, the serum levels of SOD in the aging model group were significantly lower than those in the middle and high dose groups. The serum SOD levels in the high dose group were higher than those in the low and medium dose groups. The MDA content in the serum of the aging model control group was lower, and the medium and high dose groups were increased. The MDA content in the serum of the low dose group was higher than that of the medium and high doses.

The results are not always easy to interpret. In the study,
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Interestingly, we observed that the anthocyanidin of Summer-black-grape affect the expression of Ki-67 in the testis and ovary of aging mice. Ki-67 protein is involved in the mitotic process of spermatogonia during normal testicular development in mice. It can be used as an index to evaluate the proliferative activity of testicular spermatogonia. Ki-67 protein is expressed in mouse testis all development stage, and its positive expression signal is concentrated in spermatogonia and some primary spermatocytes, and the signal in spermatogonia is stronger. The anthocyanidin group showed decreased proliferation compared to the aging group in testis. It is suggested that the anthocyanidin of Summer-black-grape promote the differentiation and maturation of cells in the gonads of aging mice. We analyzed that anthocyanidin may cause proliferation and division of cells to enter the differentiation stage, gradually differentiate into mature sperm. Then testicular reproductive function may be improved. With the anthocyanidin, the expression of Ki-67 protein in testis of aging mice decreased. In female mice, Ki-67 is mainly expressed in the nucleus of oocytes, granulosa cells and interstitial cells of primary follicles. The expression of Ki-67 protein in the ovary was decreased too. Oxidative stress is an important inducement in ovarian aging which results in fecundity decline in human and diverse animals. Zhang’s studies showed that grape seed proanthocyanidin extract treatment can effectively prevent the ovarian aging process in hens by reducing oxidative stress. Some studies showed the process of aging, few studies to date this complex process which is associated with reduced Ki-67 levels in testis and ovary. Several studies have investigated the role of oxidative stress in ovarian aging. The elderly is associated with decreased levels of antioxidants in oocytes and follicular fluids. Antioxidation can slow down the aging of ovarian tissue, consistent with our experimental results.

In summary, the anthocyanidin of Summer-black-grape have an effective anti-oxidation effect, which can reduce the MDA content in serum and increase the level of SOD and reduce the Ki-67 content. We suggest that the anthocyanidin of Summer-black-grape has anti-oxidation and anti-aging protection effect on the test ovary of aging mouse model.

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