# Effect of *Colla corii asini* (*E'jiao*) on D-Galactose Induced Aging Mice

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***Colla corii asini* (*E'jiao*), donkey-hide gelatin prepared by stewing and concentrating from *Equus asinus* L. donkey hide, is a traditional Chinese medicine preparation widely used in clinical hematic antanemic therapy in China. The aim of the present study was to investigate potential anti-aging effect of *Colla corii asini* and explore related mechanisms in D-galactose (gal) induced aging model mice. The mice were artificially induced aging by subcutaneously injection with D-gal at the dose of 100 mg/kg·d for 8 weeks. *Colla corii asini* was simultaneously treated to them once daily by intragastric gavage. Appetite, mental condition, body weight, and organ index were observed. Activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), as well as levels of malondialdehyde (MDA) in serum, brain, and liver were determined by according assay kits. Western blotting analysis was used to detect p16 and p21 expression. Results indicated that *Colla corii asini* could improve appetite, mental condition, body weight, and organ condition of model mice, improve SOD, CAT, and GSH-Px activities, reduce MDA levels, and modulate age-related genes expression in D-gal induced mice. Therefore, *Colla corii asini* may have effect to suppress the aging process through enhancing antioxidant activity, scavenging free radicals, and modulating aging-related gene expression.**

**Key words** *Colla corii asini* (*E'jiao*); anti-aging; D-galactose; antioxidant; aging-related gene

Aging is a natural phenomenon, and it is always associated with diverse chronic diseases, including cancer, Parkinson's and cardiovascular diseases, *etc*.[1,2] Anti-aging has already become a major public issue with the increasing elderly population in the world. The free radical theory of aging was conceived by Harman in 1956.[3] Abundant evidences suggest that oxidative stress plays a central role in the process of biological aging.[4] Oxygen-derived free radicals exert detrimental effects on human, including peroxidation of membrane lipids, enzyme inactivation, DNA fragmentation, and activation of apoptosis.[5] In addition, supplementation with antioxidants has been reported to be beneficial with respect to slowing this aging process.

D-Galactose (D-gal) has been used to induce oxidative stress *in vivo* to mimic the natural aging in mice. D-Gal can be metabolized at normal concentration, but when at high levels, it can be converted into galactitol under the catalysis of gal oxidase, resulting in the generation of superoxide anions and oxygen-derived free radicals.[6] As one of the antioxidant defense systems, a group of enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), act as superoxide anion and $H_2O_2$ scavengers to prevent reactive oxygen species (ROS)-induced damage, which may cause the changes of some biomarkers.[7] Malondialdehyde (MDA) is a major biomarker that appears during the final stages of lipid peroxidation initiated by excessive ROS. An increase in the hepatic MDA concentration suggests the occurrence of lipid peroxidation, tissue damage, and failure of the antioxidant defense system to prevent the formation of excessive free radicals.[8]

In recent years, numerous traditional Chinese medicines have been found to possess potent anti-aging activities, and have attracted considerable interest as potential candidates for the development of novel anti-aging therapies.[9,10] *Colla corii asini* (*E'jiao*), donkey-hide gelatin prepared by stewing and concentrating from *Equus asinus* L. donkey hide, is a traditional Chinese medicinal preparation widely used in clinical hematic antanemic therapy in China.[11] The main components were collagen, amino acids, trace elements, and so on.[12,13] It has a variety of clinical functions in terms of hemostasis, anti-fatigue, suppressing tumor growth, improving immunity, gynecologic diseases, and so on.[14,15] In addition, *Colla corii asini* has always been considered to have anti-aging effect in China. However, little study to date has addressed the effect of *Colla corii asini* on the aging process. The aim of the present study was therefore to make use of the D-gal induced aging model mice to investigate the anti-aging effects of *Colla corii asini in vivo* and explore the underlying anti-aging molecular mechanisms.

## MATERIALS AND METHODS

**Reagents** *Colla corii asini* (Lot No.: 100346) was supplied by Shandong Dong-E-E-Jiao Co., Ltd. (Dong'e, China). The amino acids contents in *Colla corii asini* were assayed using Hitachi automatic amino acid analyzer L-8900. Tryptophan was hydrolyzed with sodium hydroxide. Cysteine was treated with performic acid oxidation. All other amino acids were hydrolyzed using 6 mol/L hydrochloric acid.

Commercial antioxidant assay kits for measuring SOD, CAT, GSH-Px, and MDA were purchased from KeyGEN Biotechnology Development Co., Ltd. (Nanjing, China). Primary antibodies of p16, p21, and $\beta$-actin were purchased from Santa Cruz Biotechnology (CA, U.S.A.). Horseradish peroxidase (HRP)-labeled secondary antibodies were purchased from KeyGEN Biotechnology Development Co., Ltd. (Nanjing, China). The purity of other chemicals was of either analytical or chromatographic grade.

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**Animals and Treatments** Animal housing and all experimental procedures followed the requirements of the 'Provisions and General Recommendations of Chinese Experimental Animal Administration Legislation.' Seven-week-old male Kun-Ming mice (20±2 g) were purchased from the Experimental Animal Center of Shandong University (Jinan, China) and housed under standard conditions (22±2°C and 12 h light/dark cycle). During the entire experiment process, they had free access to food and water.

After one-week acclimatization to the home cage, the mice were randomly divided into three groups, each consists of 12 animals.

(I) Model Control Group: The mice were injected subcutaneously with D-gal at the dose of 100 mg/kg·d, and given simultaneously distilled water by intragastric gavage;

(II) Young Control Group: The mice were injected subcutaneously with the same volume of normal saline, and given simultaneously distilled water by intragastric gavage;

(III) *Colla corii asini* (0.5 g/kg·d) Group: The mice were injected subcutaneously with D-gal at the dose of 100 mg/kg·d, and given simultaneously *Colla corii asini* (0.5 g/kg·d) by intragastric gavage.

The mice were sacrificed after treatment for 8 weeks.

**Observation of General Appearance, Body Weight Measurement, and Organ Indexes** During the entire experiment process, general appearance was observed daily. After 8 weeks of administration, the mice were weighed and the blood was collected from the retro-bulbar venous plexus. After the mice were executed, the spleens, thymus glands, kidneys and livers were weighted and their weights relative to the final body weight (organ indexes) were calculated. Then, the brain and liver were stored immediately at −80°C for biochemical measurements.

**Antioxidant Measurements in Different Tissues** Blood samples were allowed to clot for 2–3 h, and the serums were separated by centrifugation at 2200×***g*** for 10 min and stored at 4°C for biochemical analysis. The tissue samples were homogenized in certain volume (10% w/v) icy 50 mM phosphate buffer (pH 7.4). After centrifugation at 3000 ×***g*** at 4°C for 10 min, the supernatants were collected for biochemical analysis. The protein concentrations were measured by BCA (bicinchoninic acid) method using bovine serum albumin as a standard. The activities of SOD, CAT, and GSH-Px, as well as the levels of MDA in serum, brain, and liver, were determined according to the assay kit providers' instructions.

**Western Blot Analysis** The tissue proteins were separated by electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to polyvinylidene difluoride membranes using a semidry transfer system. The membranes were first incubated in blocking solution (5% skim milk) and then incubated overnight at 4°C with the primary antibodies of p16 or p21. After washing with TBS-T (Tris buffered saline with Tween-20), the membranes were incubated with HRP-labeled secondary immunoglobulin G (IgG) antibodies and washed with TBS-T. Finally, immune-reactive bands were detected with HRP staining. $\beta$-Actin was served as protein loading control.

**Statistical Analysis** The results were expressed as the mean±S.D. Statistical analysis was performed by one-way analysis of variance (ANOVA) using SPSS.15.0 software. A $p<0.05$ was indicative of significant difference.

## RESULTS

**Content of Amino Acids in *Colla corii asini*** Table 1 showed the major amino acids contents of *Colla corii asini*. Glycine is the most amino acid which accounted for above 18% of *Colla corii asini*. The total amino acids contents were 73.51%.

**Effect of *Colla corii asini* on General Appearance, Body Weights, and Organ Indexes** During the entire experiment process, the aging model mice were habituated to subcutaneously (s.c.) injection with D-gal at dose of 100 mg/kg·d, and general appearance was observed daily. Observations showed that, compared with that of young control mice, the appetite and mental condition of model control mice decreased, and administration of *Colla corii asini* could attenuate these decreases.

After 8 weeks of administration, the mice were weighed and sacrificed at the indicated time. The tissues were collected for organ indexes. As shown in Table 2, mean body weight of model control mice (24.52±5.51 g) was significantly lower than that in the young control group (32.04±1.91 g) and *Colla corii asini* treatment group (34.32±5.07 g) ($p<0.05$). Meanwhile, organ indexes, including kidney, liver, thymus, and spleen, showed significant decrease in the aging model mice compared with those in young control and *Colla corii asini* treatment mice ($p<0.05$).

These results suggested that *Colla corii asini* could improve appetite, mental condition, body weight, and organ condition of D-gal induced mice.

**Effect of *Colla corii asini* on SOD Activity** SOD plays a crucial role in the balance of oxidation and antioxidation. As shown in Fig. 1, the SOD activities in serum (67.09±9.15 U/mL), brain (58.69±6.84 U/mg prot), and liver (180.85±8.36 U/mg prot) of model control mice decreased significantly ($p<0.05$) compared with young control (129.37±11.46 U/mL, 86.02±8.65 U/mg prot, and 252.09±6.22 U/mg prot) and *Colla corii asini* treatment groups (101.16±7.13 U/mL, 72.06±6.84 U/mg prot, and 336.84±9.15 U/mg prot). The results suggested

Table 1. Contents of Amino Acids in *Colla corii asini*

| Amino acids | Content (g/100 g) |
|---|---|
| Asp | 4.74 |
| Thr | 1.29 |
| Ser | 2.86 |
| Glu | 7.99 |
| Gly | 18.54 |
| Ala | 5.96 |
| Val | 2.29 |
| Met | 0.46 |
| Ile | 1.06 |
| Leu | 2.74 |
| Tyr | 0.88 |
| Phe | 1.41 |
| Lys | 2.96 |
| $NH_3$ | 3.27 |
| His | 0.56 |
| Arg | 6.14 |
| Pro | 9.56 |
| Trp | 0.50 |
| Cys | 0.30 |

Table 2. Effect of *Colla corii asini* on Organ Indexes of Mice ($\bar{x} \pm s, n = 10$)

| Group | Body weight (g) | Kidney index | Liver index | Thymus index | Spleen index |
|---|---|---|---|---|---|
| Model control | 24.52±5.51 | 12.24±1.35 | 39.89±8.34 | 1.27±0.34 | 2.69±0.75 |
| Young control | 32.04±1.91* | 13.26±0.87* | 45.64±7.26* | 1.53±0.29* | 3.14±0.56* |
| *E'Jiao* treatment | 34.32±5.07* | 13.56±0.78* | 45.59±7.89* | 2.13±0.39* | 3.42±0.38* |

*$p<0.05$, compared with the model control group.

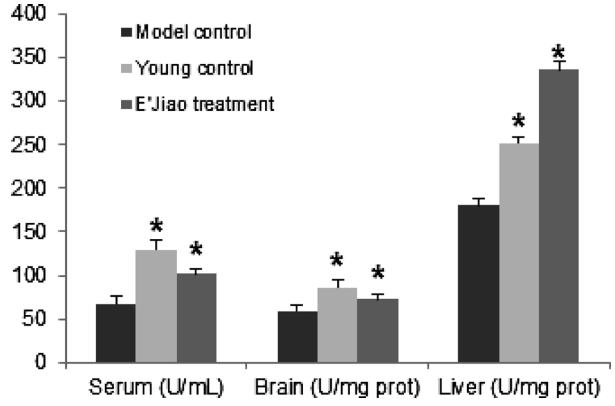


Fig. 1. Effect of *Colla corii asini* on the Activity of SOD in Serum, Brain, and Liver of D-Gal Induced Mice

Each value is the mean±S.D., $n$=10 mice. *$p<0.05$, compared with model control group.

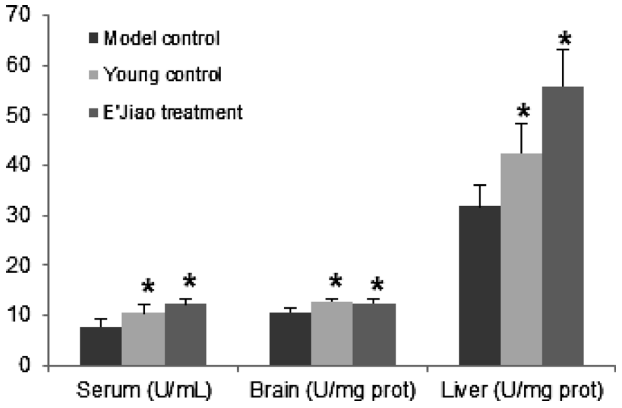


Fig. 2. Effect of *Colla corii asini* on the Activity of CAT in the Serum, Brain, and Liver of D-Gal Induced Mice

Each value is the mean±S.D., $n$=10 mice. *$p<0.05$, compared with model control group.

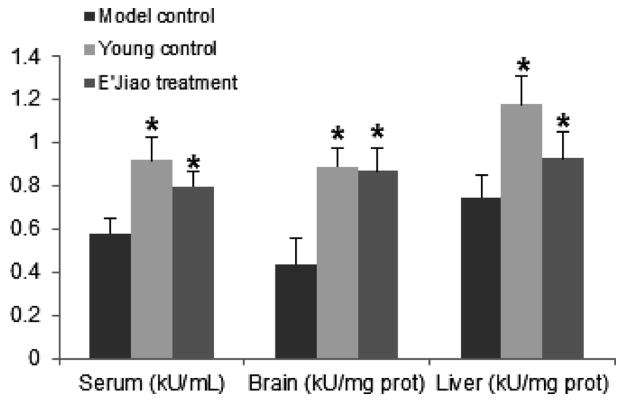


Fig. 3. Effect of *Colla corii asini* on the Activity of GSH-Px in the Serum, Brain, and Liver of D-Gal Induced Mice

Each value is the mean±S.D., $n$=10 mice. *$p<0.05$, compared with model control group.

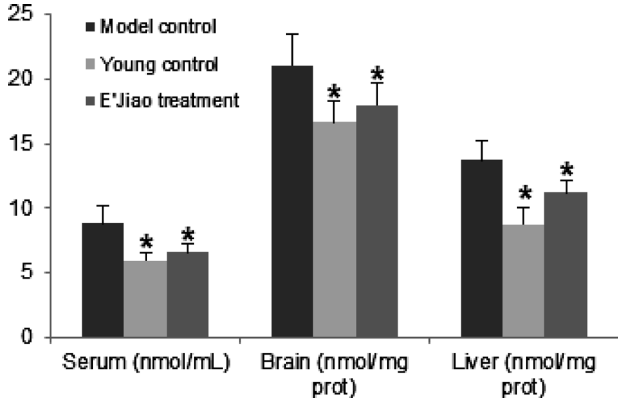


Fig. 4. Effect of *Colla corii asini* on the Levels of MDA in Serum, Brain, and Liver of D-Gal Induced Mice

Each value is the mean±S.D., $n$=10 mice. *$p<0.05$, compared with model control group.

that *Colla corii asini* could improve SOD activities in D-gal induced mice.

**Effect of *Colla corii asini* on CAT Activity** As shown in Fig. 2, CAT activities in the serum (7.82±1.31 U/mL), brain (10.69±0.92 U/mg prot), and liver (31.75±4.32 U/mg prot) of the aging model mice were reduced remarkably ($p<0.05$) compared with young control (10.38±1.91 U/mL, 12.71±0.69 U/mg prot, and 42.53±5.78 U/mg prot) and *Colla corii asini* treatment groups (12.33±0.95 U/mL, 12.37±0.87 U/mg prot, and 55.82±7.43 U/mg prot). The results suggested that *Colla corii asini* could improve CAT activities in D-gal induced mice.

**Effect of *Colla corii asini* on GSH-Px Activity** Similarly, the results showed that GSH-Px activities in serum (0.58±0.07 kU/mL), brain (0.44±0.12 kU/mg prot), and liver (0.75±0.10 kU/mg prot) decreased markedly in model group compared with young control (0.92±0.11 kU/mL, 0.89±0.09 kU/mg prot, and 1.18±0.13 kU/mg prot) and *Colla corii asini* treatment groups (0.8±0.07 kU/mL, 0.87±0.11 kU/mg prot, and 0.93±0.12 kU/mg prot) ($p<0.05$, Fig. 3). The results suggested that *Colla corii asini* could improve GSH-Px activities in D-gal induced mice.

**Effect of *Colla corii asini* on the Levels of MDA** Peroxidative damage to cellular lipid constituents was evaluated on the basis of the change of MDA content. The results showed that the model control group had a significant increase in the MDA concentrations in all serum (8.85±1.36 nmol/mL), brain (21.07±2.42 nmol/mg prot), and liver (13.75±1.53 nmol/mg

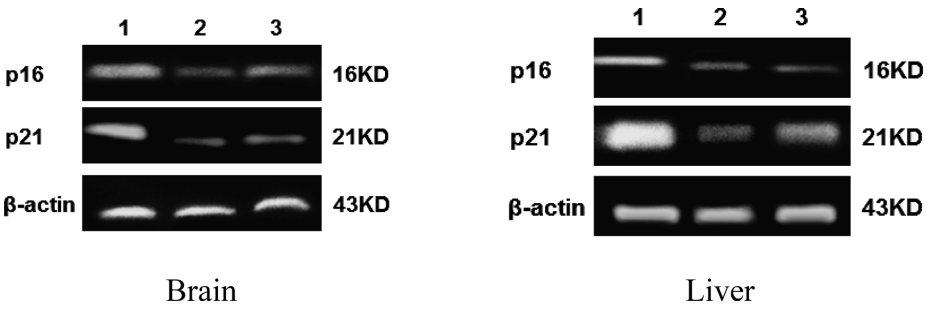


Fig. 5. Effect of *Colla corii asini* on the Expression of p16 and p21 in the Brain and Liver of D-Gal Induced Mice
(1) Model control group; (2) Young control group; (3) *Colla corii asini* treatment group.

prot) compared with the young control group (5.93±0.65 nmol/mL, 16.67±1.67 nmol/mg prot, and 8.75±1.33 nmol/mg prot) ($p<0.05$, Fig. 4), confirming that D-gal could induce the lipid oxidation. *Colla corii asini* could reverse the rise in MDA caused by D-gal significantly ($p<0.05$). The results suggested that *Colla corii asini* could reduce MDA levels in D-gal induced mice.

**Effect of *Colla corii asini* on the Aging-Related Gene Expression** A lot of studies have found that, many genes are closely related to mammalian aging, and among them, both p16 and p21 are well defined senescence-related genes. By Western blot analysis, there were more p16 and p21 expression in both brain and liver in D-gal induced mice (Fig. 5). After treatment by *Colla corii asini*, p16 and p21 expression was remarkably down-regulated in the organs. The results suggested that *Colla corii asini* could modulate age-related genes expression in D-gal induced mice.

## DISCUSSION

***Colla corii asini* Inhibiting the Aging Process** Accelerated senescence in mice can be induced by D-gal. It has been shown that D-gal treated mice were found similar to those in natural aging showing various aging symptoms.[16] D-Gal injection has been widely used to establish an aging model for anti-aging research.[17–20] The present study clearly demonstrated that administration of D-gal caused a severe aging-related appearance changes, including significant decrease in body weights and organ indexes. However, supplementation of *Colla corii asini* was able to partially reverse these adverse effects. Both body weights and organ indexes increased.

Kidney and liver are two important organs in detoxification system. Their functions were declined gradually due to their structure atrophy with age. Otherwise, immune system also presents physiological diminution. Meanwhile, thymus and spleen are important immune organs which show senescent signs firstly.[21] Our results showed that all kidney, liver, thymus, and spleen were atrophied in D-gal induced aging mice. *Colla corii asini* could increase these organ indexes. The data above mentioned suggested that *Colla corii asini* could suppress the aging process and its underlying molecular mechanisms were explored and described as follows.

***Colla corii asini* Enhancing the Activity of Antioxidant Systems** Human ageing has been believed to be an irreversible and detrimental process counted by the advance of calendar years leading finally to death.[22] According to free radical theory of aging, senescence is the result of oxidative stress.[23] A large number of studies revealed that the balance between ROS system and antioxidation system determines the degree of oxidative stress.[24] SOD serves as the first gatekeeper in the antioxidant defense system to scavenge superoxide anion free radicals. It catalyzes the dismutation of superoxide anion to oxygen and hydrogen peroxide ($H_2O_2$), and the latter is catalyzed next by CAT and/or GSH-Px as electron donor. With aging, the activities of SOD, CAT, and GSH-Px decrease. Meanwhile, the chain reaction of lipid peroxidation accelerates. MDA level usually reflects degree of lipid peroxidation and means indirect impairment level of cell.[25] The present research revealed that administration of D-gal caused the oxidative stress, decreased the antioxidant enzyme activity, and increased the MDA level. Supplementation of *Colla corii asini* could restore the antioxidant defense system by increasing the activity of antioxidant enzymes. Therefore, *Colla corii asini* will have the potential to be further explored as an antioxidant functional medicine in the prevention of aging-related diseases.

***Colla corii asini* Regulating the Aging-Related Gene Expression** Many genes are closely related to mammalian aging. P16 and p21, the two inhibitors of cell cycle progression, would increase with age and contribute to the impaired cellular regeneration of an aging organism.[26,27] P16 and/or p21 deficiency partially prevented the age-induced decline in cell proliferation and tissue function.[28] In this study, we found that *Colla corii asini* could remarkably decrease p16 and p21 expression. The results suggested that *Colla corii asini* could modulate age-related gene expression.

## CONCLUSION

In conclusion, the results mentioned above demonstrated that *Colla corii asini* could improve cognitive aging deficit induced by D-gal injection in mice. This effect may be mediated, at least partly, through enhancing antioxidant activity, scavenging free radicals, and modulating aging-related gene expression. These data suggest that *Colla corii asini* has anti-aging effect, while further longer-term investigations should be conducted to substantiate its anti-aging action and anti-aging chemical bases.

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