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# Long-term Supplementation of *Epimedium koreanum* Nakai in Rats and Its Effects on *In Vivo* Antioxidant Status with Age

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**Abstract** In this study, we investigated the effects by age of long-term supplementation of *Epimedium koreanum* Nakai (EKN)-containing water on the *in vivo* antioxidant capacities of rats. All rats were reared in a conventional system, and none of the rats showed any signs of aversion to the EKN solution. Neither the mean nor maximum life spans of the rats were extended by long-term administration of the solution. The EKN extract caused decreases in the levels of serum thiobarbituric acid reactive substances in the rats. The activities of superoxide dismutase, catalase, and glutathione (GSH) peroxidase within the liver cytosol decreased with age in both the control and EKN-supplemented groups. GSH peroxidase activity, however, was higher at old age in the EKN-supplemented group. The activities of GSH reductase and GSH-S-transferase, and the levels of free-sulfhydryl (SH) and total-SH group gradually decreased with age in both groups. However, there was some tendency for higher levels in the EKN supplemented group at a corresponding age. These results indicate that long-term supplementation of EKN water extracts alone does not exhibit discernible adverse effects in rats, and has some enhancing effects on the antioxidant capacities of the blood and liver, but it does not have life-prolonging effects.

**Keywords:** *Epimedium koreanum Nakai*, rat, aging, antioxidant status, blood, liver

## Introduction

The Orient, including Korea, China, and Japan, has respected aging since ancient times, and has made efforts to find natural pharmaceuticals for perennial youth and macrobiosis, and freedom from disease. *Epimedium koreanum* Nakai (EKN: called *umyangkwak*) is a perennial herb that belongs to the Korean barberry, and is highly valued as an oriental pharmaceutical for increasing vigor, removing paralysis, improving diuresis, and calming emotions (1). The efficacy of EKN is well-described in various ancient oriental books on medical cures, such as *Dongeubogam*, *Hyangagdaesajun*, and *Hyangyagji-bsungbang*. EKN has been reported to alleviate sharp side pains of the waists and knee pain, and to increase virility in men and birth rates in women (2). It has been known to promote the secretion of sperm in semen and to stimulate a sensory nerve to fill the spermatic fluid of the spermatic sac. It is also known to lower high blood pressure and blood glucose levels, and to have small diuresis effects and large antibiosis effects (2, 3). In addition, it is cited as being effective for curing amnesia, tinnitus, arthralgia, and especially premature ejaculation (3, 4).

Today, research papers on the efficacy of Chinese herbs are increasing due to the joining of Chinese medicine and the Western sciences such as biochemistry, analytical chemistry, pharmacology, and physiology (5-8). In particular, components of EKN have been identified by instrumental chemical analysis, and major component is reported as prenylflavone glycoside. Constituents such as icarin, icariside A, 2',5-dirhamnosyl icariside A, 2'-rhamnosyl icariside II, 4'-methoxy-5-hydroxy-8,3-dimethyl flavone, and homogeneous glucosides from EKN were also identified and reported (9, 10). In addition, a polysaccharide with a molecular weight of 75,000 Da was separated and is acknowledged as a substance with medicinal value. Prenyl flavonoids from EKN are reported to have effects on stimulating the genitalia by expanding the blood vessels in the lower abdominal region (11). Icarin has been known to generate T-suppressive cells, to increase antibodies, and to induce lymphocytes by mitogens in rats (9). EKN water-extract is reported as having an effect on raising phagocytosis functions and antioxidant actions within *in vitro* and *in vivo* systems (12, 13). Tea and extracts made with EKN can be purchased in Korea, and many people use them habitually for their invigorating effect. The efficiency of EKN is identified in folk remedies according to ancient Korean books, in chemical analyses by its diverse and effective ingredients, and in short-term biological tests by cell cultures or experimental animal systems. However, there is little data on long-term supplementation of EKN water extract in rats, from weaning to death.

Therefore, to estimate EKN's efficacy as a crude anti-aging drug preparation with possible life-prolonging effects, we investigated *in vivo* antioxidant status with age in rats by supplying EKN water extract at a rate of 25 mg/kg in drinking water from 6 weeks of age until death.

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## Materials and Methods

**Chemicals** Ferricytochrome C, NADPH, reduced glutathione (GSH), oxidized glutathione (GSSG), GSH reductase, xanthine oxidase, hydrogen peroxide, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), cumene hydroperoxide, bovine serum albumin (BSA), 1-chloro-2,4-dinitrobenzene (CDNB), thiobabituric acid (TBA), N-[2-hydroxyethyl]piperazine-N'-[2-ethansulfonic acid] (Hepes),

and potassium chloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of the highest grade.

**Preparation of EKN water extract** EKN was purchased by consultation with the herbology laboratory at the Department of Oriental Medicine in Daejeon University. It was sliced to 2 cm of thickness and ground by a mixer (30-40 mesh). The water extract was made from the EKN powder by soaking it in 5 vol. (v/w) hot water for 4 hr. The temperature of the water was maintained at 70°C to prevent heat destruction of the saponins and other phenolic compounds (14). This procedure was repeated more than twice. The extracts were combined, concentrated to a gel state, and stored at 16% water content until use.

**Animals and EKN supplementation** Sprague-Dawley (SD) rats were bred in an animal house, and only the males were used as experimental animals in this study. To keep individual differences at a minimum, healthy 6 weeks old SD rats (50±3 g) were selected and housed singly in polycarbonate cages. All rats were reared in a conventional system with cycles of 12 hr at 200-300 Lux and 12 hr of darkness. The animals were maintained at a temperature of 22±1°C and 40-60% humidity with complete air exchange 15 times per hr. The animals were given solid feed purchased from Sam Yang Co. (Seoul, Korea) *ad libitum*. Food intake and the consumption of drinking water were checked regularly every morning. There were 72 animals total, and each had free access to the EKN water extract (25 mg/kg body weight/day) in drinking water from 6 weeks of age; an equal number of rats in the control group were given only drinking water. The EKN water extract was replaced every day for freshness. This treatment continued throughout their natural life, or until an animal was sacrificed for biochemical assays.

Cross-sectional studies were carried out on a fraction of the rats, using 32 rats from the control group and 32 rats from the EKN supplemented group. Eight rats from each of the 2 groups were sacrificed by decapitation at 3, 6, 12, and 24 months of age. The remaining 40 animals in each group were utilized for the longevity study.

**Oxidative damage in serum** Blood was collected by cardiac puncture and serum was separated by centrifugation (2,500×g, 10 min). The serum was divided into small vials and stored at -70°C until analysis. The TBA reactive substance content of the serum was determined by the method of Suematsu *et al.* (15).

**Antioxidant capacities of livers** The livers were removed, weighed, and washed well with saline, and then homogenized in 4 vol. of 30 mM Hepes buffer (pH 7.4) containing 150 mM KCl. The cytosolic fraction was prepared from the homogenates by differential centrifugation (16). Superoxide dismutase (SOD) activity in the cytosol was measured according to the procedure of McCord *et al.* (17) by the inhibition of ferricytochrome C oxidation at 550 nm using xanthine oxidase to generate superoxide radicals. Catalase activity was assayed by the method of Lee *et al.* (18), spectrophotometrically, based on the direct measurement of hydrogen peroxide decomposition at 240 nm. GSH peroxidase activity was measured with the coupled-enzyme system (18). The reduction of GSSG was coupled to NADPH oxidation by GSH peroxidase, using cumene hydroperoxide as substrate. GSH reductase activity was determined by the method of Racker (19). The oxidation of NADPH was monitored at 340 nm. Glutathione-S-transferase (GST) activity was assayed by the method of Habig *et al.* (20) using CDNB as substrate. Total SH-groups content was measured at 412 nm according to the procedure of Sedlak and Lindsay (21) using DTNB. Protein concentration was determined by the method of Lowry *et al.* (22) with BSA as the standard.

**Statistical analysis** Data are expressed as mean ± standard deviation (SD). The data differences between the control group and the EKN supplemented group were determined using analysis of variance (StatView version 4.0; Abacus Concepts, Inc., Berkeley, CA, USA). If the differences between two groups were statistically significant ($p<0.05$), Fisher's protected least significant difference test or Scheffe's F test were used to distinguish between pairs of groups.

## Results and Discussion

**Side effect** During the last decade there has been growing concern regarding the benefits and safety of herbal and other natural products (dietary supplements) as an increasing number of Americans, as well as Asians, are using herbal products for preventive and therapeutic purposes (23, 24). Subsequently, many researchers have taken issue with the safety and adverse effects of herbal products (25). In Korea especially, herbal medicines are viewed not as medical cures, but as preventive medicines, and many take them more often than necessity requires, without verification of the subsidiary ill effects. Accordingly, our results furnish new meaningful information about the side effects of herbal products. Namely, there were no remarkable symptoms in rats from the long-term supplementation of EKN water extract. The actual amount of EKN supplemented to the rats in this study corresponds to 1.5 g of dry EKN powder per 60 kg of body weight per day. These data indicate that EKN water extracts of this concentration do not have any side effects on rats over time.

**Longevity** Figure 1 shows the survival curve for the rats. The mean life span of the control rats was 656±88 days and maximum life span was 837 days. For the EKN supplemented rats, the mean and maximum life-spans were 635±100 and 832 days, respectively. The maximum life-spans in the two groups were similar, but the mean life span of the control group was somewhat longer than in the EKN supplemented group, although there was no significant statistical difference between the two groups.

In general, the life-spans of rodents demonstrate large differences according to strain and rearing conditions. It has been known for 70 years that restricting the food intake of laboratory rats extends their mean and maximum life spans (26). The underlying biological mechanism responsible for this life extension is still unknown,

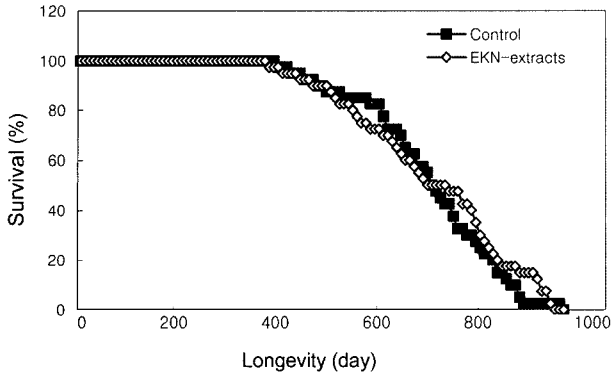


**Fig. 1. Survival curve for control and EKN supplemented rats.** Male S.D. rats received drinking water with (EKN) or without (control) EKN water extract. Forty rats per group were utilized for the longevity study.

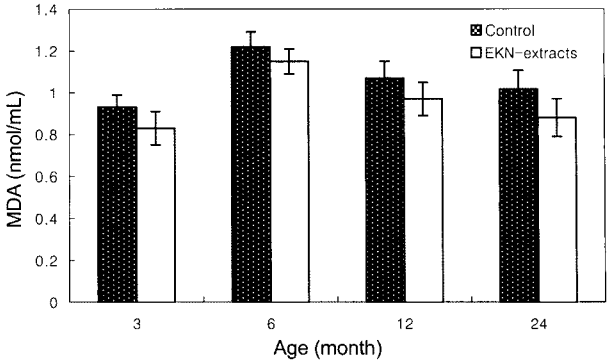


**Fig. 2. Changes in the content of serum TBA reactive substances with age in control and EKN supplemented rats.** Male S.D. rats received drinking water with (EKN) or without (control) EKN water extract. Values are expressed as mean±SD from 8 male S.D. rats.

although many hypotheses have been proposed. It has been suggested that EKN can increase long-term resistance to stress and disease, and therefore, affect life-span. We set out to investigate this idea by testing whether the continuous supplementation of EKN water extract from weaning to spontaneous death could affect the life-span of rats. We observed no differences in appearance between the EKN supplemented group and the control group. The supplemented rats were generally healthy and stable throughout their lives, similar to the control group. However, the long-term supplementation of EKN in the rats did not have a statistically significant life-extending effect on either the mean or maximum life-span.

**Oxidative damage in serum** Diverse oxygen free radicals are produced during oxygen consumption and are removed by precise defense mechanisms in the body. An imbalance between the formation and removal of oxygen free radicals is known to give rise to various degenerative diseases by oxidizing biomolecules of cell membranes and organ tissues (27, 28). Since TBA reactive substances in serum reflect the status of oxidative stress in the whole body, they are used as sensitive markers of oxidative stress *in vivo*. We determined the content of serum TBA reactive substances, and the results are presented in Fig. 2. Levels of serum TBA reactive substances increased at 6 months old, decreased at 12 months, and were the same at 24 months as compared with 3 months; a similar pattern by age was shown in both groups. Conflicting data exist concerning serum TBA reactive substance concentrations, with increases (29) or no change (30) in old humans and rodents; although most papers have generally shown an age-related increase in aging rodents. Nakamura *et al.* (31) demonstrated that the level of TBA reactive substances and antioxidant status in serum are not affected by age, but by the ratio of vitamin E to vitamin C. Interestingly, TBA reactive substances had a tendency to be maintained at low levels in the EKN supplemented rats, compared with the controls, throughout their whole lives.

**Antioxidant capacities in livers** Antioxidant components and enzymes in the liver protect the cells and tissues against attacks by xenobiotics and oxygen free radicals, and play important roles in maintaining homeostasis. Antioxidant enzymes such as SOD, catalase, GSH peroxidase, and GSH reductase are located in the cytosol, and are directly and indirectly involved in the aging process by protecting living bodies from oxidative stress (32). The changes in SOD activities in the liver cytosol with age by EKN extract supplementation are presented in Fig. 3. The SOD activities in the control group decreased with age and were maintained at a level of 62.4% at 24 months of age compared to 3 months of age. SOD activity in the EKN-supplemented group also decreased with age, but it was maintained at a level of 79.3% at 24 months of age. In order to compare the decomposing efficiency of organic peroxide within the livers of the two groups, the levels of catalase and GSH peroxidase, major antioxidant enzymes, were determined by age and are presented in Table 1. There was a significant decrease in the catalase activity of both groups with age ($p<0.01$). The level of this enzyme was maintained at 55.6% at 24 months and 61.3% at 3 months in the control and EKN supplemented groups, respectively. The enzyme's activity was somewhat higher

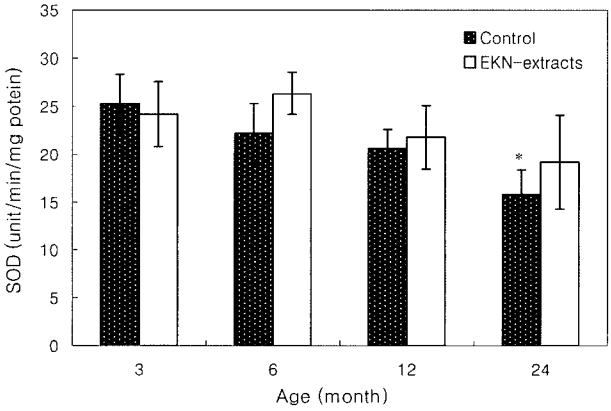


**Fig. 3. Age related changes of SOD activities in the livers of control and EKN supplemented rats.** Male S.D. rats received drinking water with (EKN) or without (control) EKN water extract. Values are expressed as mean±SD from 8 male S.D. rats. Significantly different from control rats at the same age ($p<0.05$).

**Table 1. Age-related changes of catalase and GSH peroxidase activities in livers of control and EKN supplemented rats**

| Age (month) | Catalase[1)] | | GSH peroxidase[1)] | |
|---|---|---|---|---|
| | Control | EKN | Control | EKN |
| 3 | 201.6±12.4 | 214.5±9.9 | 2.46±0.13 | 2.41±0.23 |
| 6 | 190.4±23.4 | 197.0±20.8 | 2.15±0.18 | 2.62±0.25 |
| 12 | 170.4±21.7 | 189.7±27.6 | 2.26±0.21 | 2.71±0.19[#3)] |
| 24 | 112.1±9.4[*2)] | 131.4±19.3[*] | 1.44±0.15[*] | 1.89±0.26[*#] |

[1)]μmole/min/mg protein; Male S.D. rats received drinking water with (EKN) or without (control) EKN water extract (25 mg/kg body weight); Each point is the mean±SD of 8 male S.D. rats.
[2)]*Significantly different from 3 months of age ($p<0.01$).
[3)]#Significantly different from the control rats at the same age ($p<0.05$).

**Table 3. Age-related changes in the contents of total-SH and free-SH groups in livers of control and EKN supplemented rats**

| Age (month) | T-SH[1)] | | F-SH[1)] | |
|---|---|---|---|---|
| | Control | EKN | Control | EKN |
| 3 | 11.4±0.7 | 12.1±0.5 | 2.83±0.43 | 2.96±0.55 |
| 6 | 13.3±0.8 | 12.9±1.3 | 3.11±0.66 | 3.22±0.80 |
| 12 | 15.0±1.0 | 15.4±1.6 | 4.66±1.41 | 4.93±1.12 |
| 24 | 10.2±0.8 | 12.2±1.6 | 3.44±0.61 | 3.81±0.60 |

[1)]μmole/g tissue of liver; Male S.D. rats received drinking water with (EKN) or without (control) EKN water extract. Each point is the mean± SD of 8 male S.D. rats.

in the EKN supplemented group than in the control group at corresponding ages, without any statistical significance. In the EKN supplemented rats, GSH peroxidase activity of the liver decreased age-dependently. However, it was the best preserved enzyme at old age among the three antioxidant enzymes. The preservation of GSH peroxidase activity in old age may contribute largely to the elimination of toxic organic peroxides. This seems to be closely related to the trend of the TBA reactive substances in serum.

GSH reductase is associated with the GSH/GSSG cycle in cells. In normal cells, various kinds of peroxides that form can be scavenged because the ratio of GSH/GSSG is high. But if the GSH/GSSG ratio is lowered by oxidative stress, GSSG, which is oxidized GSH, reacts readily with the protein of SH groups, and mixed disulfide bonds are formed allowing the protein to be denaturated (33). GST plays an important role in detoxifying oxygen free radical generators and metabolized materials by combining with GSH as one of the phase II enzymes of metabolism in the body (32). The age-related changes of GSH reductase and GST activities in the livers of rats are presented in Table 2. GSH reductase activity in the control group had a tendency to decrease with age, and clearly decreased with age in the EKN supplemented group ($p<0.01$). There were no apparent differences shown at corresponding ages between the two groups. GST activities declined in old age in both groups ($p<0.01$), and a significant difference between the two groups was not shown at all ages. The enzyme's activity was somewhat higher in the EKN supplemented group than in the control group. SH groups such as GSH detoxify xenobiotics directly in the body and protect from DNA damage caused by radioactive materials. In particular, GSH acts as the substrate for GSH peroxidase and GST, and contributes to peroxide removal and xenobiotic biotransformation (33). Table 3 shows the changes in total-SH and free-SH contents with age in the liver. Cytosolic concentrations of total-SH and free-SH in both groups had increasing trends from 6 to 12 months of age, and then declined at 24 months of age. Overall, concentrations varied with age and there were no clear differences between the two groups at corresponding ages.

**Table 2. Age-related changes of GSH reductase and GST activities in livers of control and EKN supplemented rats[1)]**

| Age (month) | GSH reductase[2)] | | GST[3)] | |
|---|---|---|---|---|
| | Control | EKN | Control | EKN |
| 3 | 30.9±2.7 | 32.7±2.3 | 1008±47 | 1054±57 |
| 6 | 31.7±6.8 | 27.7±4.3 | 1005±103 | 973±52 |
| 12 | 26.1±5.3 | 24.0±5.6 | 962±72 | 1079±98 |
| 24 | 25.1±6.3 | 23.2± .3[*4)] | 687±35[*] | 733±70[*] |

[1)]Male S.D. rats received drinking water with (EKN) or without (control) EKN water extract; each point is the mean±SD of 8 male S.D. rats.
[2)]μmole/min/mg protein.
[3)]nmole/min/mg protein.
[4)]Significantly different from 3 months of age ($p<0.01$).

Animals live a normal sexual life like human beings. We can not know whether increased sexual appetite by EKN supplementation, as referred to in ancient oriental books, had any important effect on the experimental results since we used only males in this study. Unlike in animals, immunity and resistance against disease may be affected by the mind in human beings. Also, Oriental herbal medicine is built of common knowledge focusing on human beings as a science, and created by experience upon experience from immemorial antiquity; it is not free from side effects, or the effects of having to take it for a long time. Accordingly, the problem that awaits a solution is whether these results apply to human beings directly. Therefore, these results can offer suggestions from a visual standpoint, but more research is necessary for their further application.

In conclusion, our study demonstrated that long-term supplementation of EKN water extract alone, from weaning to death, had no side effects in rats. On the other hand, it offered some enhancing effects on the antioxidant capacities of the blood and liver, but did not influence either the mean or maximum life-spans.

## Acknowledgments

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