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Safety Evaluation of *Epimedium koreanum* Water Extract in Sprague-Dawley Rats

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Abstract : To evaluate of oral toxicity of *Epimedium koreanum nakai* (EKN) water extract, three doses of EKN water extract (10, 100 and 1000 mg/kg/day) were administered to healthy Sprague-Dawley rats for 4 weeks. The results showed that there are no abnormal signs during the experimental period. The weights of testis and epididymis were increased dose-dependently, but seminal vesicle was decreased compared with control group. In hematology and serum chemistry, changes were observed within the normal ranges. In histological finding, slight to moderated changes were found but those were commonly or rarely encountered in the normal rats. These results suggest that no observed adverse effect level (NOAEL) of the oral application of EKN was considered to be more than 1000 mg/kg in SD rats under the conditions employed in this study.

Key words **:** *Epimedium koreanum*, Safety evaluation, Rat.

Introduction

Epimedium Koreanum nakakai (EKN) has been traditionally used as diuretic, tonic, antirheumatic and remedy of impotency and menstrual disorder in the oriental medicine (14). Lately, researchers ascertained many medicinal components such as prenylflavonol glycoside (8,23), icariin (13,32,33), magnoflorine (6), chlorogenic acid (31), korepimedoside A and B (34), hyperin (30), and so on. This components have many effects on anti-oxidant (15,18,21), anti-cancer in human lung cancer cells and prostate carcinoma (22,36), anti-osteoporosis by stimulating proliferation of bone marrow stromal cells (5,37), stimulation of erectile function of penis (5,20), anti-stress by decreasing brain monoamine oxidase A and B, and serum corticotropin-releasing factor levels, and increasing brain monoamine neurotransmitter levels in rats and mice (25,26).

Although many researchers have interested in icariin's effect as natural stimulant for the sex drive, but the toxicological aspects of the medicinal herbs have been neglected because these plants have been used for a long time. As regards with EKN, there are few reports concerning its toxicological aspects. The purpose of this study, therefore, is to know the possible toxicity of the EKN water extract by orally 4 weeks consecutive administration.

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Materials and Methods

Epimedium koreanum nakai (EKN)

The EKN were obtained from herbal market in Daegu, Korea. The leaves of EKN were ground and the powder extracted with distilled water by water bath (140rpm, 37°C) for 4 hours. The aqueous solution was centrifuged, filtered, concentrated and lyophilized. The extract was stored at -20°C until the use.

Animals

Twenty, 3-months-old male SD rats (355 ± 24 g BW) were purchased from Orient Bio inc. (Seoul) and acclimated for 1 week. The rats were housed in a room with humidity of 50 ± 10% at 20 ± 1°C, under 12 hours-intervals of light and dark cycle, and food and water were supplied *ad libitum*.

Experimental design

All experiment was conducted under the procedures according to the Guide for the Care and Use of Laboratory Animals of the Korea Food and Drug Administration (Seoul, 2005). The rats were divided into three experimental groups and one control group (n=5). The rats of experimental groups (EKN-10, -100, -1000) were administered with 10, 100 and 1,000 mg/kg/day of the EKN water extract, *per os*, using gastric gavage daily for 4 weeks, respectively. Control group was given with saline in the same manner.

Sample collection

After 4 weeks administration, all rats were anesthetized and bled via caudal vena cava after measure of the body weight, and the blood samples were subjected to hematological analysis by automatic analyzer (MS9-5, Kubota, Japan). The rats were sacrificed by cervical dislocation and organs, such as heart, lung, spleen, liver, thymus, stomach, left kidney, bladder, left testis, left epididymis, prostate and lefts seminal vesicle were dissected and weighed after washing with phosphate buffered saline (PBS) and wiped out PBS from organs. These organs were subjected to histological examination. The right testis and epididymis were stored at -20°C and used for calculation of daily sperm production and total sperm count, respectively.

Blood cell count and serum chemistry

The blood samples were mixed with EDTA-2Na in the microfuge tube and used for complete blood cell (CBC) counts using automatic hematologic analyzer (MS9-5, Kubota, Japan), and portion of the blood was mixed with heparin in the microfuge tube and plasma was obtained by centrifugation at 3,000 rpm for 20 min. Serum chemistrical values such as aspartate aminotrasferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin (T-bil), creatinine and blood urine nitrogen (BUN) were analyzed using dry chemistry-analyzer (Spotchem™) (SP-4410, Kyoto Daiichi Kagaku, Japan).

Daily sperm production from testis and sperm count in cauda epididymis

Daily sperm production (DSP) was examined from the right testis according to the methods of Blazak *et al* (3). Briefly, frozen testis was thawed on ice and weighed after decapsulation, and homogenized in 50 ml of Hank's solution. The homogenate was allowed to settle down tissue debris for 1 min, and the supernatant sperm was decanted to a new tube. The sperm solution was evenly suspended and 10 ml-aliquot of the solution was collected into a glass vial and stored on ice. After thorough mixing, the number of sperm heads was counted by hemocytometer. The number of sperms produced per gram of testicular tissue was calculated according to the following formula: the average count of sperm heads in four chambers × square factor [5] × hemocytometer factor [10^4] × dilution factor [50] divided by testis weight [g] and the time of 6.1 days (28) in which the sperm is undamaged by homogenization after spermatogenesis.

To count the sperm number in the cauda epididymis, 0.5 ml of homogenate of right cauda epididymis were diluted with 4.5 ml of Hank's solution. The total number of sperm heads was counted using a hemocytometer, and the mean of three calculations per gram of cauda epididymis was calculated (28,38).

Histological examination

Tissues of heart, lung, liver, stomach, kidney, bladder, prostate and seminal vesicle were fixed in Bouin solution, and those of thymus, spleen, testis and epididymis were fixed in 10% neutral buffered formalin. All these tissues were embedded in paraffin, sectioned (3-4 μm) and stained with hematoxylin and eosin, and observed under light microscope.

Statistical analysis

All data were expressed with means ± standard deviation. The values of body weight, organ weight, CBC count, serum chemistry, DSP and sperm count were analyzed using student *t* test. The values significantly different with those of control group were indicated at the levels of $P < 0.05$ and $P < 0.01$.

Results

After administration of EKN water extract for 4 weeks, the body weights of EKN-10, -100 and -1000 were increased 0%, 3.6%, -10.7% compared with control group, respectively. But there are no significant changes in the body weight. In the aspects of organ weights, EKN-10 group has significant ($P < 0.05$) changed in stomach (A: absolute, R: relative), spleen (A), thymus (A,R) and lung (R). EKN-100 has significant ($P < 0.05$ or 0.01) changes in the bladder, thymus (R). There was no significant changes of heart, liver, kidney in all EKN groups (Table 1).

The weights of the testis, epididymis and prostate in all EKN groups were increased dose-dependently compared with control group. Especially, the absolute weights of testis in EKN-100 and EKN-1000 group were increased significantly ($P < 0.05$), and the absolute weights of epididymis in EKN-100 group were also increased significantly ($P < 0.05$). But weight of the seminal vesicle decreased in all EKN groups compared with control group. In relative weighs of these organs, there were no significant changes (Table 2).

The red blood cell(RBC) counts in all EKN groups were increased compared with control group, but the increase was dose-independent. Also, the hemoglobin in EKN-10 and EKN-1000 groups were increased significantly ($P < 0.01$), and the packed cell volume (PCV) and platelet in EKN-1000 was increased significantly ($P < 0.05$). On the other hand, the mean corpuscular hemoglobin concentration in EKN-10 group were increased significantly ($P < 0.01$), while in EKN-1000 group was decreased significantly ($P < 0.01$) and the mean corpuscular volume in EKN-1000 group was increased significantly ($P < 0.01$), while that in EKN-10 and EKN-100 groups were decreased significantly with $P < 0.01$ and $P < 0.05$, respectively. The white blood cell (WBC) of EKN-10 and EKN-1000 groups were also decreased significantly ($P < 0.01$). There was no significant change in mean corpuscular hemoglobin in all EKN groups (Table 3).

The ALT, ALP, and BUN levels of EKN-1000 groups were decreased significantly ($P < 0.05$) compared with control group. T-bil in EKN-1000 group was higher than normal range, whereas the AST and creatinine in all EKN groups ranged in normal values (Table 4).

The weights and DSP of testis were increased dose-depen-

Table 1. Changes of organ weights after administration of EKN water extract in SD rats

Items \ Groups	Control	A dose of EKN water extract administered [a]		
		EKN-10	EKN-100	EKN-1000
Body weight (g)				
Initial	350 ± 22[b]	350 ± 14	350 ± 20	340 ± 12
Final	420 ± 29	420 ± 8	423 ± 19	403 ± 36
Increase ratio(%) *vs* control	-	0	3.6	-10.7
Absolute organ weight (g)				
Heart	1.25 ± 0.15	1.27 ± 0.17	1.21 ± 0.03	1.17 ± 0.05
Lung	1.27 ± 0.09	1.48 ± 0.20	1.56 ± 0.39	1.31 ± 0.21
Liver	15.15 ± 0.99	14.85 ± 1.70	15.57 ± 1.28	13.23 ± 0.94
Stomach	1.78 ± 0.13	2.15 ± 0.18*	1.99 ± 0.19	1.71 ± 0.06
Spleen	0.75 ± 0.07	0.66 ± 0.05*	0.71 ± 0.10	0.55 ± 0.22
Kidney	1.53 ± 0.16	1.56 ± 0.06	1.65 ± 0.16	1.43 ± 0.10
Bladder	0.13 ± 0.03	0.12 ± 0.02	0.14 ± 0.02*	0.12 ± 0.01
Thymus	0.24 ± 0.03	0.28 ± 0.03*	0.27 ± 0.02	0.24 ± 0.07
Relative organ weight[c] (%)				
Heart	0.29 ± 0.04	0.30 ± 0.04	0.28 ± 0.02	0.30 ± 0.03
Lung	0.29 ± 0.02	0.35 ± 0.04*	0.36 ± 0.10	0.33 ± 0.07
Liver	3.46 ± 0.48	3.52 ± 0.37	3.62 ± 0.45	3.33 ± 0.44
Stomach	0.42 ± 0.05	0.51 ± 0.23*	0.46 ± 0.07	0.43 ± 0.05
Spleen	0.18 ± 0.02	0.16 ± 0.01	0.16 ± 0.02	0.17 ± 0.03
Kidney	0.35 ± 0.05	0.37 ± 0.02	0.38 ± 0.05	0.36 ± 0.05
Bladder	0.03 ± 0.01	0.03 ± 0.00	0.03 ± 0.01	0.03 ± 0.00
Thymus	0.05 ± 0.01	0.07 ± 0.03*	0.07 ± 0.01**	0.06 ± 0.02

[a]; each of 10, 100 and 1,000 mg/kg/day of EKN water extract was administered each 5 rats orally for 28 days. [b]; The values were expressed with means ± SD. [C]; Ratio of the organ weight divided by body weight.
*, **: each of indicated $P < 0.05$ and $P < 0.01$ compared to that of control group, respectively.

Table 2. Changes of reproductive organ weights after administration of EKN water extract in SD rats

Items \ Groups	Control	A dose of EKN water extract administered [a]		
		EKN-10	EKN-100	EKN-1000
Absolute organ weight (g)				
Testis	1.44 ± 0.04 [b]	1.47 ± 0.13	1.55 ± 0.09*	1.56 ± 0.12*
Epididymis	0.55 ± 0.03	0.60 ± 0.05	0.61 ± 0.03*	0.58 ± 0.04
Prostate	0.88 ± 0.14	1.04 ± 0.21	1.03 ± 0.19	1.04 ± 0.12
Seminal vesicle	0.86 ± 0.07	0.70 ± 0.25	0.76 ± 0.24	0.64 ± 0.27
Relative organ weight[c] (%)				
Testis	0.33 ± 0.02	0.35 ± 0.03	0.36 ± 0.02	0.39 ± 0.06
Epididymis	0.13 ± 0.01	0.14 ± 0.01	0.14 ± 0.01	0.15 ± 0.02
Prostate	0.22 ± 0.03	0.25 ± 0.05	0.24 ± 0.05	0.26 ± 0.04
Seminal vesicle	0.20 ± 0.03	0.17 ± 0.06	0.18 ± 0.06	0.16 ± 0.07

[a]; each of 10, 100 and 1,000 mg/kg/day of EKN water extract was administered each 5 rats orally for 28 days. [b]; The values were expressed with means ± SD. [C]; Ratio of the organ weight divided by body weight.
*; indicated $P < 0.05$ compared to that of control group.

dently in all EKN groups, although these values in EKN-10 and EKN-100 groups were lower than those in control group. The sperm counts of EKN-10 and EKN-1000 groups were also increased in cauda epididymis (Table 5).

In the lung of EKN-10 group, there were hypertrophy of alvelous walls, focal infiltrations of inflammatory cells, exists

Table 3. Changes of complete blood cell counts after administration of EKN water extract in SD rats

Items \ Groups	Control	A dose of EKN water extract administered [a]		
		EKN-10	EKN-100	EKN-1000
RBC($\times 10^6$/mm^3)	6.67 ± 0.36[b]	7.24 ± 0.33**	7.18 ± 0.23	7.15 ± 0.38
Hb(g/dl)	13.30 ± 0.20	14.44 ± 0.59**	13.95 ± 0.72	14.30 ± 0.82**
PCV(%)	36.02 ± 1.96	36.18 ± 1.61	36.43 ± 1.50	40.62 ± 3.10*
MCV(fl)	54.04 ± 1.82	50.02 ± 1.79**	50.70 ± 1.42*	56.74 ± 2.10**
MCH(pg)	19.98 ± 0.86	19.96 ± 0.71	19.43 ± 0.63	19.98 ± 0.26
MCHC(g/dl)	37.02 ± 1.65	39.88 ± 0.25**	38.33 ± 0.43	35.22 ± 1.47**
WBC($\times 10^3$/mm^3)	15.60 ± 2.60	8.56 ± 1.46**	11.66 ± 4.73	6.35 ± 2.58**
Platelet($\times 10^3$/mm^3)	562.20 ± 72.38	513.40 ± 47.56	530.50 ± 131.93	712.20 ± 80.14*

[a]; each of 10, 100 and 1,000 mg/kg/day of EKN water extract was administered each 5 rats orally for 28 days. [b]; The values were expressed with means ± SD.
*, **: each of indicated $P < 0.05$ and $P < 0.01$ compared to that of control group, respectively.

Table 4. Changes of serum chemistry values after administration of EKN water extract in SD rats

Items	Control	A dose of EKN water extract administered [a]		
		EKN-10	EKN-100	EKN-1000
AST (IU/l)	76.40 ± 14.94[b]	73.80 ± 21.68	81.25 ± 28.04	77.00 ± 15.22
ALT (IU/l)	25.60 ± 4.51	23.60 ± 7.73	26.75 ± 6.60	20.20 ± 4.21*
ALP (IU/l)	574.60 ± 84.67	603.40 ± 125.17	509.75 ± 163.96	415.00 ± 110.55*
T-bilirubin (mg/dl)	0.22 ± 0.04	0.20 ± 0.00	0.23 ± 0.05	0.64 ± 0.93
Creatinine (mg/dl)	0.66 ± 0.09	0.68 ± 0.08	0.75 ± 0.06	0.70 ± 0.17
BUN (mg/dl)	23.20 ± 2.17	20.60 ± 3.21	21.50 ± 2.08	19.40 ± 1.34*

[a]; each of 10, 100 and 1,000 mg/kg/day of EKN water extract was administered each 5 rats orally for 28 days. [b]; The values were expressed with means ± SD.
*, **: each of indicated $P < 0.05$ and $P < 0.01$ compared to that of control group, respectively.

Table 5. Changes of daily sperm production of testis and sperm counts of cauda epididymis after administration of EKN water extract in the SD rats

Items \ Groups	Control	A dose of EKN water extract administered [a]		
		EKN-10	EKN-100	EKN-1000
Testis				
Weight (g)	1.39 ± 0.12[b]	1.28 ± 0.02	1.38 ± 0.11	1.42 ± 0.11
DSP ($\times 10^8$/g)	5.90 ± 0.80	5.62 ± 0.06	5.77 ± 0.50	5.90 ± 0.27
Epididymis				
Weight (g)	0.23 ± 0.03	0.21 ± 0.03	0.23 ± 0.02	0.23 ± 0.03
Sperm count ($\times 10^8$/g)	15.99 ± 0.86	17.29 ± 4.03	15.48 ± 2.08	16.62 ± 3.57

[a]; each of 10, 100 and 1,000 mg/kg/day of EKN water extract was administered each 5 rats orally for 28 days. [b]; The values were expressed with means ± SD.
*, **: each of indicated $P < 0.05$ and $P < 0.01$ compared to that of control group, respectively.

of exudate in the lumen and focal hemorrhages, a classic aspiration congestions, as slight (1+) morphological change as in one rat of the control group. In addition, moderate (2+) congestion was found in one rat of the control group. Slight hepatic fatty changes were observed in two rats of EKN-10 and one rat of EKN-1000 groups as in two rats of the control group. Slight erosive/ulcerative lesions on the mucosa of the stomach were observed in one rat of EKN-100 and two rats of EKN-1000 groups, as in two rats of the control group. Focal tubular necrosis on the kidney was observed in one of EKN-10 and one rat of EKN-100 groups, as in a rat of the control group. Slight depletions of thymic lymphoid cells were observed in two rats of EKN-10 group. Only a rat of the control group showed focal inflammatory cell infiltration in prostate (Table 6).

Meanwhile, no histopathological lesions were observed in

Table 6. Histopathological findings after administration of EKN water extract in SD rats

Items \ Groups	Control	A dose of EKN water extract administered[a]		
		EKN-10	EKN-100	EKN-1000
Heart normal	5/5[b]	5/5	4/4	5/5
Lung - left lobes				
normal	3/5	4/5	4/4	5/5
congestion 1+	1/5	1/5	0/4	0/5
congestion 2+	1/5	0/5	0/4	0/5
Liver - left lateral lobes				
normal	3/5	3/5	4/4	4/5
focal fatty changes 1+	2/5	2/5	0/4	1/5
Stomach - fundus				
normal	3/5	5/5	3/4	3/5
focal erosive/ulcerative lesion 1+	2/5	0/5	1/4	2/5
Spleen				
normal	5/5	/5	4/4	5/5
Kidney-left				
normal	4/5	4/5	3/4	5/5
focal tubular necrosis 1+	1/5	1/5	1/4	0/5
Bladder				
normal	5/5	5/5	4/4	5/5
Thymus				
normal	5/5	3/5	4/4	5/5
depletion of lymphoid cells 1+	0/5	2/5	0/4	0/5
Testis - left				
normal	5/5	5/5	4/4	5/5
Epididymis-head left				
normal	5/5	5/5	4/4	5/5
Prostate				
normal	4/5	5/5	4/4	5/5
focal inflammatory cells 1+	1/5	0/5	0/4	0/5
Seminal vesicle				
Normal	5/5	5/5	4/4	5/5

[a]; each of 10, 100 and 1,000 mg/kg of aqueous EK extract was administered each 5 rats orally for 28days.
[b]; Observed animals/total observed animals; Degree, 3+ sever, 2+ moderate, 1+ slight.

heart, spleen, bladder, testis, epididymis and seminal vesicle in all EKN groups (Table 6).

Discussion

In the present study, we investigated the subacute toxicity of the EKN water extract in SD rats. The animals were received repeated doses of EKN water extract at 10, 100 and 1000 mg/kg/day for 4 weeks consecutive days. During the experimental period, no mortality and signs of toxicity were observed in the EKN water extract dosing groups, as compared with the control group.

Body weights of all EKN groups were comparable to control group, but slightly decreased in EKN-1000 group without any statistical significance.

Organ weight is a simple, sensitive index of toxicity after exposure to toxic substances (35). Weight of some organs, such as stomach, spleen, thymus, bladder, were significantly ($P < 0.01$ or $P < 0.05$) changed compared with control group. But those changes were sporadic occurrence without dose-dependant. The other hands, in reproductive organ, weight of the seminal vesicle were decreased in all EKN-groups, the weight of reproductive organs such as testis and epididymis in all EKN groups were increased dose-dependently, and were significantly ($P < 0.05$) increased in testis of EKN-100 and -1000 group, and epididymis in EKN-100 compared with control group.

Lee (19) reported that weights of liver, spleen and kidney were increased by drinking water containing 0.025% EKN extract. Especially, that of testis was increased until 12-months-old, whereas that was decreased over 12-months-old. This would explain that the EKN has positive effects on the male reproductive organs.

Biochemical parameters are an important maker to evalu-

ate the organ and cellular functions (10,24). The RBC was increased dose-independently in all EKN groups. Moreover, the Hemoglobin in EKN-10 and -1000 group, and the PCV in EKN-1000 group was increased significantly with $P < 0.01$ and $P < 0.05$, respectively. In the case of WBC, there were significant decrease ($P < 0.01$) in EKN-10 and -1000 group compared with control group. But those changes were within normal ranges and not dose-dependant. As like CBC value changes, some values in serum chemistry were significantly ($P < 0.01$ or $P < 0.05$) changed but mostly were changed within normal ranges.

In fecundity, although DSP of testis was decreased compared with control group, DSP of all EKN dosing groups was increased dose-dependently. Interestingly, sperm count of cauda epididymis was increased compared with control group. This is contrary result of Zhen (39)'s study that is icariin has testosterone mimetic properties. More study is needed comparing activated complex and single substance (icariin).

In histological aspects, although there were slight to moderate morphological changes randomly found in organs of some EKN groups, such as lung, liver, stomach, kidney and thymus, these were observed as the lesions as those frequently appeared in the control group with similar degrees and frequencies. Therefore, these signs were considered as accidental findings rather than toxicological signs from EKN administration. In addition, these lesions were not found in any dose-dependent distributions and degrees, and most of them were commonly or rarely encountered in the healthy normal rat (1,2,4,7,9,11,12,16,17,27,29). Thus, no histopathological changes related to EKN administration were observed after oral administration for 4 weeks in the present study. On the other hand, other organs, such as heart, spleen, bladder, testis, epididymis and seminal vesicle in all EKN groups had no histological changes. Therefore, it was recognized that the EKN had no histopathological toxicity in rats.

Consequently, base on the results in the present study, it was concluded that oral administration of EKN water extract in the concentration up to 1000 mg/kg/day for 4 weeks had shown no toxicity in rats. Also, it was uncertain but recognized that the EKN revealed accelerating effects on the male reproductive organs, meanwhile it had no adverse effects on the values of blood cell count and biochemistry in SD rats.

Conclusion

This present study concludes that the oral administration of the EKN water extract, at 10, 100 and 1000 mg/kg/day for 4 weeks to male SD rats, did not induce any toxicological effects. The NOAEL of EKN water extract in SD rat was determined to be 1000 mg/kg/day over.

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음양곽 물추출물의 독성 평가

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요 약 : 음양곽 물추출물의 랫에서의 안전성을 검증하기 위하여 그룹당 5마리씩 총 20마리의 수컷 SD 랫에 각각 음양곽 물추출물을 체중당 10, 100, 1000 mg/kg을 1일 1회 4주간 경구투여 하였다. 대조군으로는 생리식염수를 동량 투여하였다. 임상증상, 체중 및 사료섭취량, 혈액, 혈액생화학 및 조직학적 검사를 실시하였다. 시험기간 중 임상증상, 체중, 사료섭취량의 이상변화를 관찰할 수 없었으며, 투약종료 다음날 부검결과 고환, 부고환의 무게가 용량의존적으로 증가하였으나, 정낭선은 감소경향을 나타내었다. 조직학적 검사결과 일부 변화가 관찰되었으나 건강한 쥐에서도 발견되는 변화였다. 따라서 체중당 1000 mg까지의 투여는 임상적으로 안전한 것으로 사료된다.

주요어 : 음양곽, 독성평가, 랫드.