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Original Article

Single-dose and 4-week repeated dose Toxicity of Aconitum Sinomontanum Nakai Pharmacopuncture: An Experimental Study

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ABSTRACT

Background: This study aimed to assess the toxicity of Aconitum sinomontanum Nakai (ASN) pharmacopuncture.
Methods: To investigate the toxicity of ASN pharmacopuncture, single and 4-week repeated dose toxicity experiments were conducted on BALB/c mice. In the single-dose toxicity experiment, mice were assigned 1 of 4 groups (5 males, 5 females per group). Then, 31.25, 62.5, and 125 mg/kg of ASN pharmacopuncture were administered to the mice in the experimental groups at acupoint ST36, while 0.2 mL of normal saline was administered to the control group at ST36. After a 4-week repeated dose regimen, the mice were assigned into 4 groups (5 males, 5 females per group). Then, 15.625, 31.25, and 62.5 mg/kg of ASN pharmacopuncture at ST36 were administered to the mice in the experimental groups, while 0.2 mL of normal saline was administered to the control group at ST36. Mortality, morbidity, general body and organ weight changes (after 4 weeks repeated dose), serum hematological and biochemical values, and histopathological changes in the liver and kidney were observed.
Results: In both single and 4-week repeated dose toxicity experiments, no deaths or symptoms occurred in any of the groups. There were no significant differences between groups in terms of body and organ weights, serum hematological and biochemical values, and specific organ histopathological changes.
Conclusion: ASN pharmacopuncture injection did not demonstrate significant toxicity in BALB/c mice compared with the control group, with a no-observed-adverse-effect level for a single dose of >125 mg/kg, and for 4 weeks repeated dose it was more than 62.5 mg/kg/day.

Introduction

Pharmacopuncture combines herbal medicine and acupuncture which are directly injected into the acupoint that needs to be treated on the basis of the properties-flavors theory of traditional Chinese medicine. This therapy joins meridian and herb factors and may enhance the delivery of herbal medicines to target sites [1]. However, it is important to consider that herbal medications used in pharmacopuncture are natural substances and may be subject to different manufacturing processes which could amount to different levels of the active ingredients being present in a preparation [2].

Aconitum sinomontanum Nakai (ASN) is a root belonging to the family Ranunculaceae, which is usually cultivated in the central and western regions of China [3]. The main ingredients of ASN are lappaconine, ranaconitine, appaconitine, 8-O-acetylexcelsine, N-deacetyllappaconitine, N-deacetylranaconitine. ASN is known to have anti-inflammatory and analgesic properties [4].

ASN drugs manufactured in Korea and used for pharmacopuncture have been reported to control pain [5]. Sprague-Dawley rats were injected with ASN up to 500 mg/kg without reducing mortality. Hence, it was not possible to determine the lethal dose 50 % (LD50). Localized skin rashes occurred with a dose of 500 mg/kg in rats [6].

However, there is no long-term study on ASN pharmacopuncture toxicity. Therefore, to safely use ASN pharmacopuncture in the clinical setting, a toxicological study on ASN after repeated therapy was conducted in BALB/c mice for 4 weeks.

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Materials and Methods

Experimental animals

Male and female BALB/c mice (weighing 30 ± 1 g; Samtako bio Korea Osan-si, Gyeonggi-do, Korea) were housed in standardized cages with ventilation under standard conditions (temperature: 23-24°C; relative humidity: 40-60%) and 12 hour dark/light cycle. The mice had free access to water and food. After an acclimatization period of 7 days, the mice were randomly distributed into study groups. All procedures were conducted according to guidelines by the Animal Research Ethics Committee of Dong-eui University, Korea (no.: A2019-013).

Preparation of ASN pharmacopuncture

ASN (300 g of finely ground powder; Sichuan Province, China), was diluted to a 10 % concentration in 90 % EtOH solution, and heated using a heating mantle (DMS637, MTOPS, Seoul, South Korea) for 2 hours at 80°C, this process was repeated then the solution was filtered through the reflux extraction process. The solution was alkalinized at pH > 10 using ammonium hydroxide and the extract (5.2 L) was dissolved in chloroform (5.2 L). The solution was vacuumed and concentrated using a rotary evaporator (R-3, BUCHI, Flawil, St.Gallen, Switzerland) and the residue obtained was dissolved in acetone which was re-crystallized with diethyl ether.

Single-dose toxicity study

For the single-dose toxicity study, BALB/c mice were randomly assigned into 3 equal groups: low (31.25 mg/kg), intermediate (62.5 mg/kg), and high concentration (125 mg/kg). There was no statistical differences in the characteristics of the mice, and ASN pharmacopuncture or 0.2 mL of saline (control group) was injected once on the right leg (acupoint ST36).

Four-week repeated toxicity study

A 4-week repeated toxicity study was performed on 3 groups of mice: low 15.625 mg/kg, intermediate (31.25 mg/kg), and high concentration (62.5 mg/kg) groups. Five BALB/c mice were randomly assigned to each group, and ASN pharmacopuncture or 0.2 mL of saline (control group) was injected once a day on the right foot area (ST36). Injections were given to the mice every day at approximately 10 $_{A.M.}$ for 4 weeks and after which the animals were ethically euthanizing and treatment toxicity was observed.

Observation of general symptoms

The animals' health status was monitored for up to 30 minutes after ASN pharmacopuncture at both single and 4-week repeated toxicity studies i.e., loss of fur, soft stools or diarrhea, polyuria, motor activity, tremor, edema, and death. The condition of the mice was also observed after 1, 2, 4, and 6 hours after the procedure. In the subsequent 4 weeks of repeated toxicity testing, the condition of the mice were checked more than once a day from the 1st day of the procedure until the end of the experiment.

Weight measurement

In the 4-week repeated toxicity experiment, the weights of the mice were measured before, and 7, 14, 21, and 28 (autopsy) days after the procedure.

Blood tests

In the single and 4-week repeated toxicity experiments, the mice had nothing orally for approximately 12 hours before being euthanized with ethyl ether and cervical dislocation was carried out. Blood of each dead animal was treated with an anticoagulant (ethylenediaminetetraacetic acid) and measurements taken following Hemavet parameters (Drew Scientific Co., Miami Lakes, Florida, USA) to measure white blood cells, red blood cells, hemoglobin, hematocrit, platelets, mean cell volume, mean cell hemoglobin, and mean cell hemoglobin concentration.

Blood biochemical tests

After centrifugation of the blood at 3,000 rpm (Eppendorf, Hamburg, Germany) for 15 minutes, the serum was examined for the following parameters using an automatic serum analyzer (Hitachi7060, Hitachi, Tokyo, Japan): total protein, albumin, total bilirubin, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, blood urea nitrogen, creatinine, total cholesterol, and triglyceride levels.

Gross inspection of major organs and organ weight measurements

Autopsies were performed on all the mice. During the procedure, each organ was weighed and a gross inspection was performed to check for abnormalities. Organs such as the brain, heart, lungs, thymus, liver, kidneys, spleen, and reproductive organs (testes in males, ovaries in females) were removed and evaluated.

Histopathological examination

Hepatic and renal tissues were sampled for histologically through the process of fixation, embedding into paraffin, cutting into slices, and hematoxylin-eosin (H&E) staining and changes in pathology were observed.

Statistical analysis

Statistical analysis was performed using ANOVA run on statistical software (SAS Version 9.1.3; SAS Institute Inc., Cary, North Carolina, USA). The level of significance was set at $p < 0.05$. Student *t*-test was used to compare the means in each group. The figures for all values are expressed as mean ± SD.

Results

Single-toxicity test of ASN pharmacopuncture

Observation of general symptoms

Symptoms such as fur loss, soft stools and diarrhea, polyuria, soft stool, decreased motor activity, tremors, and edema did not occur in both the control and case groups after a single treatment of ASN pharmacopuncture. No mortalities occurred.

Blood test

The blood tests (white blood cells, red blood cells, hemoglobin, hematocrit, platelets, mean cell volume, mean cell hemoglobin, and mean cell hemoglobin concentration) after ASN pharmacopuncture treatment did not show any significant differences compared with the control group (Tables 1 and 2).

Blood biochemistry tests

The blood biochemistry tests showed no significant difference

Table 1. The Hematological Values of Male Mice in the Single Dose Toxicity Study of ASN Pharmacopuncture.

Index	Normal	ASN-L	ASN-M	ASN-H
White blood cell (10^3/μL)	3.18 ± 0.18	3.24 ± 0.37	3.26 ± 0.53	3.25 ± 0.12
Red blood cell (10^6/μL)	7.97 ± 0.19	8.03 ± 0.11	8.16 ± 0.19	8.04 ± 0.49
Hemoglobin (g/dL)	10.95 ± 0.95	11.0 ± 1.33	11.25 ± 0.66	11.19 ± 0.44
Hematocrit (%)	31.23 ± 2.66	30.97 ± 2.44	31 ± 2.84	31.39 ± 3.30
Platelet (10^3/μL)	939.72 ± 31.78	940.67 ± 52.67	962.92 ± 26.38	938.19 ± 44.01
Mean cell volume (fl)	42.42 ± 4.42	45.63 ± 5.27	44.07 ± 7.35	42.17 ± 5.64
Mean cell hemoglobin (pg)	12.29 ± 0.83	12.84 ± 2.37	12.63 ± 1.69	12.93 ± 0.89
Mean cell hemoglobin concentration (g/dL)	32.28 ± 4.29	32.82 ± 3.85	30.94 ± 3.37	30.34 ± 2.02

The values represent mean ± SD.
Normal: Male BALB/c mice group injected with normal saline.
ASN-L: Male BALB/c mice group injected with low dose of ASN pharmacopuncture (31.25 mg/kg).
ASN-M: Male BALB/c mice group injected with intermediate dose of ASN pharmacopuncture (62.5 mg/kg).
ASN-H: Male BALB/c mice group injected with high dose of ASN pharmacopuncture (125 mg/kg).
ASN, Aconitum sinomontanum Nakai.

Table 2. The Hematological Values of Female Mice in the Single Dose Toxicity Study of ASN Pharmacopuncture.

Index	Normal	ASN-L	ASN-M	ASN-H
White blood cell (10^3/μL)	3.36 ± 0.39	3.13 ± 0.21	3.18 ± 0.2	3.27 ± 0.24
Red blood cell (10^6/μL)	8.13 ± 0.21	8.02 ± 0.32	7.96 ± 0.56	8.18 ± 0.27
Hemoglobin (g/dL)	10.53 ± 1.43	10.82 ± 0.91	10.74 ± 0.87	10.37 ± 0.69
Hematocrit (%)	32.9 ± 1.94	32.08 ± 0.68	33.29 ± 0.74	31.97 ± 2.62
Platelet (10^3/μL)	955.48 ± 45.07	896.69 ± 19.26	930.97 ± 19.39	901.31 ± 38.24
Mean cell volume (fl)	41.73 ± 5.20	39.85 ± 5.57	40.74 ± 2.08	45.12 ± 7.79
Mean cell hemoglobin (pg)	12.63 ± 0.89	13.28 ± 1.5	14.13 ± 1.08	13.78 ± 1.54
Mean cell hemoglobin concentration (g/dL)	32.24 ± 3.67	32.64 ± 2.04	33.00 ± 3.48	31.41 ± 3.46

The values represent mean ± SD.
Normal: Female BALB/c mice group injected with normal saline.
ASN-L: Female BALB/c mice group injected with low dose of ASN pharmacopuncture (31.25 mg/kg).
ASN-M: Female BALB/c mice group injected with intermediate dose of ASN pharmacopuncture (62.5 mg/kg).
ASN-H: Female BALB/c mice group injected with high dose of ASN pharmacopuncture (125 mg/kg).
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between the ASN pharmacopuncture and control groups (Tables 3 and 4).

Changes in visual inspection and major organ weights

No significant differences were noted on gross inspection, nor were there any differences in major organ weights between the ASN pharmacopuncture and control groups (Tables 5 and 6; Figs. 1 and 2).

Histopathological changes in the liver and kidneys

Histopathological evaluation of liver tissues of the ASN pharmacopuncture group and control groups did not reveal abnormal findings (e.g., inflammation, fatty degeneration, or hepatocyte necrosis). No histopathological changes (e.g. glomerular or tubular damage) were observed in the liver tissues of the ASN pharmacopuncture and control groups, and normal structures of the portal venule, bile duct, Kupffer cells, hepatic arteriole, and hepatocytes were observed (Figs. 3 and 4).

Table 3. The Serum Biochemical Values of Male Mice in the Single Dose Toxicity Study of ASN Pharmacopuncture.

Index	Normal	ASN-L	ASN-M	ASN-H
Total protein (g/dL)	5.11 ± 0.17	4.81 ± 0.67	4.89 ± 0.38	4.92 ± 0.3
Albumin (g/dL)	1.41 ± 0.13	1.53 ± 0.07	1.48 ± 0.08	1.57 ± 0.09
Total bilirubin (mg/dL)	0.13 ± 0.04	0.11 ± 0.02	0.13 ± 0.01	0.14 ± 0.04
Alkaline phosphatase (IU/L)	39.8 ± 3.72	42.71 ± 6.99	41.91 ± 5.65	39.86 ± 5.26
Aspartate aminotransferase (IU/L)	50.82 ± 1.34	53.77 ± 9.66	46.22 ± 4.49	52.52 ± 2.56
Alanine aminotransferase (IU/L)	21.27 ± 3.9	18.3 ± 1.25	21.26 ± 1.41	18.45 ± 1.43
Creatinine (mg/dL)	0.46 ± 0.09	0.51 ± 0.10	0.44 ± 0.12	0.47 ± 0.10
Blood urea nitrogen (mg/dL)	17.45 ± 1.64	17.46 ± 1.75	18.56 ± 1.91	18.27 ± 1.95
Cholesterol (mg/dL)	145.06 ± 16.08	150.96 ± 24.51	153.92 ± 4.93	150.11 ± 14.48
Triglyceride (mg/dL)	69.79 ± 4.00	66.91 ± 5.69	71.09 ± 5.05	70.24 ± 5.11

The values represent mean ± SD.
Normal: Male BALB/c mice group injected with normal saline.
ASN-L: Male BALB/c mice group injected with low dose of ASN pharmacopuncture (31.25 mg/kg).
ASN-M: Male BALB/c mice group injected with intermediate dose of ASN pharmacopuncture(62.5 mg/kg).
ASN-H: Male BALB/c mice group injected with high dose of ASN pharmacopuncture (125 mg/kg).
ASN, Aconitum sinomontanum Nakai.

Table 4. The Serum Biochemical Values of Female Mice in the Single Dose Toxicity Study of ASN Pharmacopuncture.

Index	Normal	ASN-L	ASN-M	ASN-H
Total protein (g/dL)	5.11 ± 0.28	5.02 ± 0.38	5.22 ± 0.30	5.18 ± 0.40
Albumin (g/dL)	1.53 ± 0.08	1.54 ± 0.08	1.49 ± 0.07	1.43 ± 0.12
Total Bilirubin (mg/dL)	0.14 ± 0.02	0.13 ± 0.03	0.15 ± 0.02	0.14 ± 0.04
Alkaline phosphatase (IU/L)	42.31 ± 4.66	40.74 ± 4.9	38.96 ± 3.25	40.51 ± 5.56
Aspartate aminotransferase (IU/L)	48.81 ± 5.06	47.88 ± 3.57	50.81 ± 6.25	50.32 ± 2.88
Alanine aminotransferase (IU/L)	19.47 ± 3.18	20.30 ± 4.08	20.54 ± 2.5	18.57 ± 2.74
Creatinine (mg/dL)	0.41 ± 0.05	0.47 ± 0.11	0.54 ± 0.08	0.47 ± 0.12
Blood urea nitrogen (mg/dL)	19.99 ± 2.69	18.05 ± 1.32	19.91 ± 2.34	17.46 ± 1.18
Cholesterol (mg/dL)	147.83 ± 7.27	145.14 ± 11.77	154.52 ± 8.35	154.99 ± 18.33
Triglyceride (mg/dL)	65.60 ± 4.50	67.80 ± 6.78	66.79 ± 13.30	68.18 ± 8.38

The values represent mean ± SD.
Normal: Female BALB/c mice group injected with normal saline.
ASN-L: Female BALB/c mice group injected with low dose of ASN pharmacopuncture (31.25 mg/kg).
ASN-M: Female BALB/c mice group injected with intermediate dose of ASN pharmacopuncture (62.5 mg/kg).
ASN-H: Female BALB/c mice group injected with high dose of ASN pharmacopuncture (125 mg/kg).
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Table 5. The Relative Organ Weight Values of Male Mice in the Single Dose Toxicity Study of ASN Pharmacopuncture.

Group	Normal	ASN-L	ASN-M	ASN-H
Heart	0.13 ± 0.04	0.12 ± 0.02	0.11 ± 0.03	0.13 ± 0.03
Liver	1.27 ± 0.06	1.29 ± 0.13	1.31 ± 0.15	1.25 ± 0.1
Kidney (right)	0.24 ± 0.04	0.24 ± 0.05	0.21 ± 0.05	0.22 ± 0.02
Kidney (left)	0.24 ± 0.02	0.25 ± 0.04	0.23 ± 0.02	0.22 ± 0.02
Brain	0.4 ± 0.05	0.46 ± 0.05	0.46 ± 0.03	0.42 ± 0.02
Lung	0.25 ± 0.05	0.28 ± 0.04	0.28 ± 0.05	0.22 ± 0.02
Spleen	0.12 ± 0.03	0.11 ± 0.01	0.11 ± 0.01	0.12 ± 0.02
Thymus	0.08 ± 0.03	0.09 ± 0.01	0.09 ± 0.01	0.11 ± 0.03
Testis	0.08 ± 0.03	0.09 ± 0.03	0.07 ± 0.01	0.07 ± 0.01
Gastrocnemius	0.1 ± 0.03	0.1 ± 0.02	0.09 ± 0.04	0.1 ± 0.03

The values represent mean ± SD.
Normal: Male BALB/c mice group injected with normal saline.
ASN-L: Male BALB/c mice group injected with low dose of ASN pharmacopuncture (31.25 mg/kg).
ASN-M: Male BALB/c mice group injected with intermediate dose of ASN pharmacopuncture (62.5 mg/kg).
ASN-H: Male BALB/c mice group injected with high dose of ASN pharmacopuncture (125 mg/kg).
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Table 6. The Relative Organ Weight Values of Female Mice in the Single Dose Toxicity Study of ASN Pharmacopuncture.

Group	Normal	ASN-L	ASN-M	ASN-H
Heart	0.13 ± 0.02	0.13 ± 0.02	0.14 ± 0.02	0.11 ± 0.02
Liver	1.25 ± 0.10	1.26 ± 0.11	1.26 ± 0.11	1.29 ± 0.11
Kidney (right)	0.23 ± 0.03	0.26 ± 0.03	0.25 ± 0.02	0.24 ± 0.05
Kidney (left)	0.23 ± 0.02	0.22 ± 0.03	0.22 ± 0.02	0.23 ± 0.02
Brain	0.46 ± 0.04	0.42 ± 0.04	0.41 ± 0.02	0.44 ± 0.05
Lung	0.25 ± 0.03	0.23 ± 0.02	0.23 ± 0.02	0.26 ± 0.07
Spleen	0.14 ± 0.04	0.12 ± 0.02	0.14 ± 0.02	0.16 ± 0.04
Thymus	0.10 ± 0.02	0.10 ± 0.02	0.12 ± 0.05	0.10 ± 0.02
Ovary	0.08 ± 0.02	0.09 ± 0.01	0.09 ± 0.01	0.08 ± 0.02
Gastrocnemius	0.09 ± 0.03	0.11 ± 0.02	0.10 ± 0.03	0.10 ± 0.04

The values represent mean ± SD.
Normal: Female BALB/c mice group injected with normal saline.
ASN-L: Female BALB/c mice group injected with low dose of ASN pharmacopuncture (31.25 mg/kg).
ASN-M: Female BALB/c mice group injected with intermediate dose of ASN pharmacopuncture (62.5 mg/kg).
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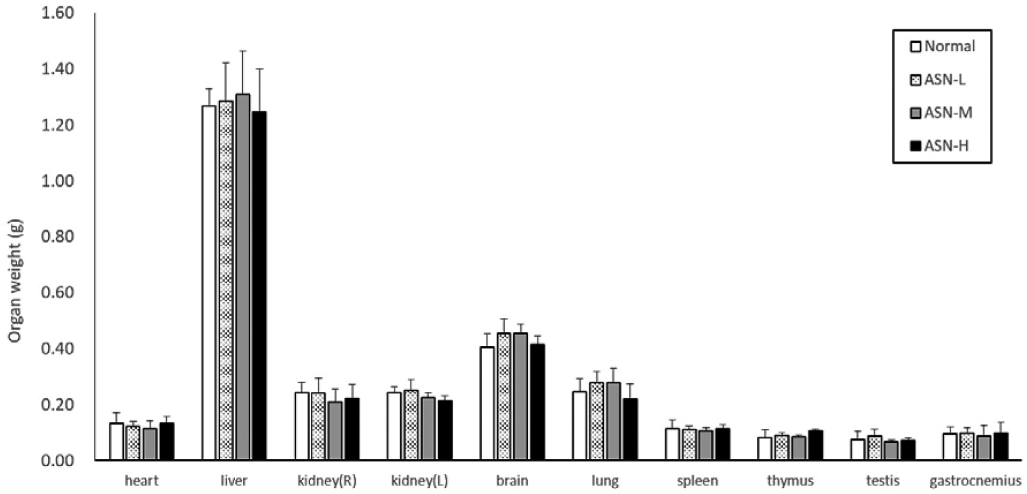

Fig. 1. The relative organ weight values of male mice in the single dose toxicity study of ASN pharmacopuncture.
The values represent mean ± SD.
Normal: Male BALB/c mice group injected with normal saline.
ASN-L: Male BALB/c mice group injected with low dose of ASN pharmacopuncture (31.25 mg/kg).
ASN-M: Male BALB/c mice group injected with intermediate dose of ASN pharmacopuncture (62.5 mg/kg).
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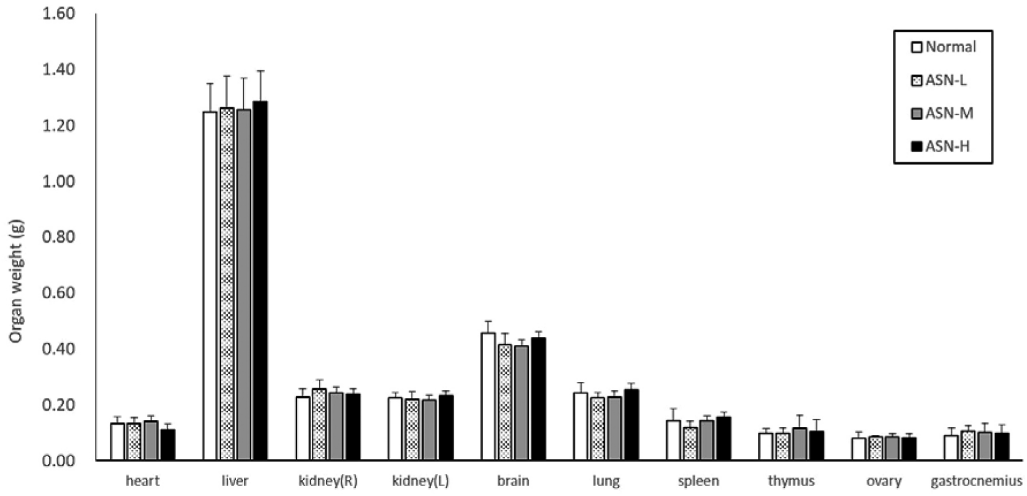

Fig. 2. The relative organ weight values of female mice in the single dose toxicity study of ASN pharmacopuncture.
The values represent mean ± SD.
Normal: Female BALB/c mice group injected with normal saline.
ASN-L: Female BALB/c mice group injected with low dose of ASN pharmacopuncture (31.25 mg/kg).
ASN-M: Female BALB/c mice group injected with intermediate dose of ASN pharmacopuncture (62.5 mg/kg).
ASN-H: Female BALB/c mice group injected with high dose of ASN pharmacopuncture (125 mg/kg).
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4-week repeated ASN pharmacopuncture toxicity test

Observation of general symptoms

Symptoms such as fur loss, soft stools, diarrhea, polyuria, decreased motor activity, tremors, and edema did not occur in either control or ASN pharmacopuncture groups. Mortality did not occur in either group.

Weight change

Normal weight gain was observed in both the control and ASN pharmacopuncture groups during the 4-week experimental period. There was no significant difference in weight gain between the 2 groups, or between male and female mice. No group had a dose-dependent change in weight due to ASN pharmacopuncture treatment (Figs. 5 and 6).

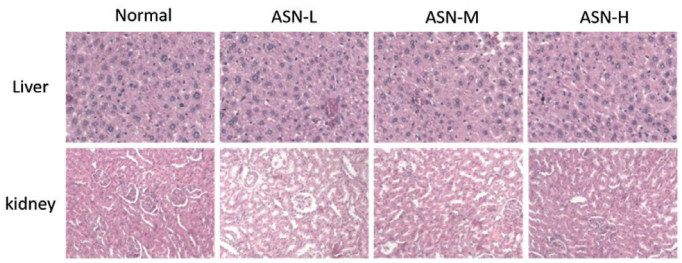

Fig. 3. Hematoxylin-eosin stained liver and kidney tissues from male mice injected once with ASN pharmacopuncture.
Normal: Male BALB/c mice group injected with normal saline.
ASN-L: Male BALB/c mice group injected with low dose of ASN pharmacopuncture (31.25 mg/kg).
ASN-M: Male BALB/c mice group injected with intermediate dose of ASN pharmacopuncture (62.5 mg/kg).
ASN-H: Male BALB/c mice group injected with high dose of ASN pharmacopuncture (125 mg/kg).
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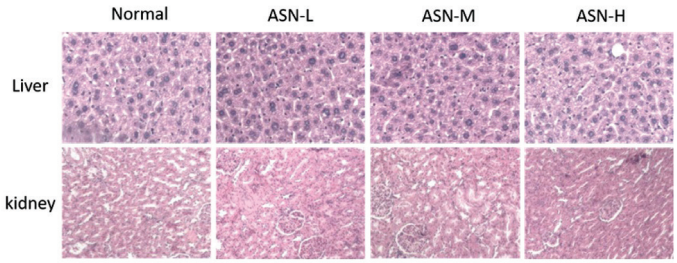

Fig. 4. Hematoxylin-eosin stained liver and kidney tissues from female mice injected once with ASN pharmacopuncture.
Normal: Female BALB/c mice group injected with normal saline.
ASN-L: Female BALB/c mice group injected with low dose of ASN pharmacopuncture (31.25 mg/kg).
ASN-M: Female BALB/c mice group injected with intermediate dose of ASN pharmacopuncture (62.5 mg/kg).
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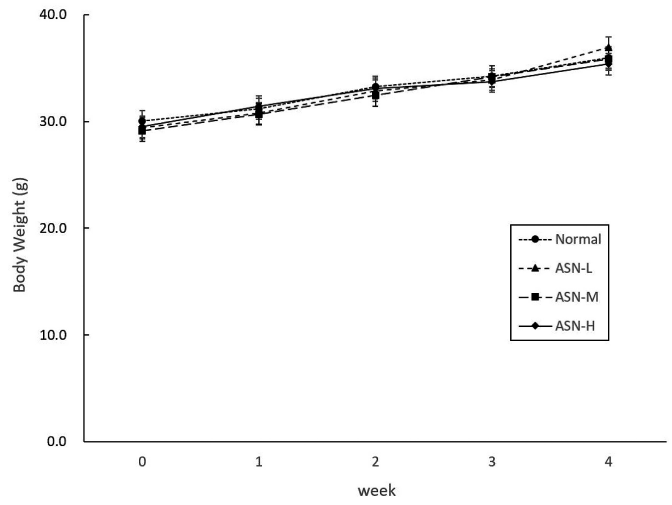

Fig. 5. Body weight changes in the male mice of each group.
The values represent mean ± SD.
Normal: Male BALB/c mice group injected with normal saline.
ASN-L: Male BALB/c mice group injected with low dose of ASN pharmacopuncture (15.63 mg/kg).
ASN-M: Male BALB/c mice group injected with intermediate dose of ASN pharmacopuncture (31.25 mg/kg).
ASN-H: Male BALB/c mice group injected with high dose of ASN pharmacopuncture (62.5 mg/kg).
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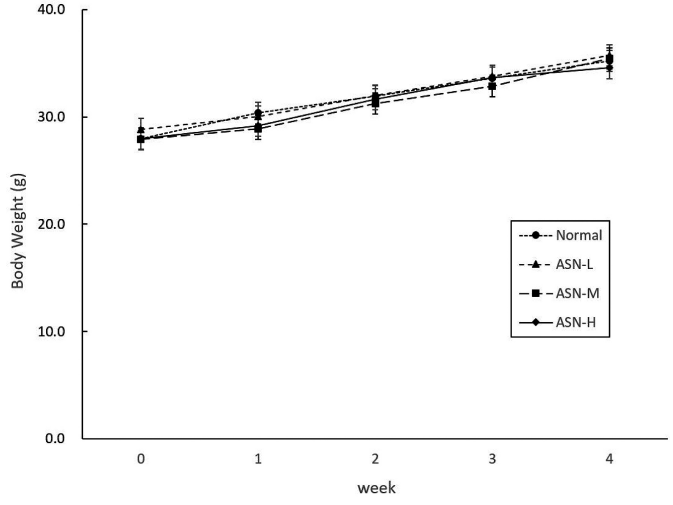

Fig. 6. Body weight changes in the female mice of each group.
The values represent mean ± SD.
Normal: Female BALB/c mice group injected with normal saline.
ASN-L: Male BALB/c mice group injected with low dose of ASN pharmacopuncture (15.63 mg/kg).
ASN-M: Male BALB/c mice group injected with intermediate dose of ASN pharmacopuncture (31.25 mg/kg).
ASN-H: Male BALB/c mice group injected with high dose of ASN pharmacopuncture (62.5 mg/kg).
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Blood tests

The results of blood tests (white blood cells, red blood cells, hemoglobin, hematocrit, platelets, mean cell volume, mean cell hemoglobin, and mean cell hemoglobin concentration) after ASN pharmacopuncture treatment did not show any significant differences between the ASN pharmacopuncture group and the control group (Tables 7 and 8).

Blood biochemistry test

The results of the blood biochemistry tests showed no significant difference between ASN pharmacopuncture and control groups (Tables 9 and 10).

Changes in visual inspection and major organ weights

There were no significant changes in gross inspection and major organ weights in both ASN pharmacopuncture and control groups (Tables 11 and 12; Figs. 7 and 8).

Histopathological changes in the liver and kidneys

The histopathological evaluation of liver tissues of the ASN

pharmacopuncture groups did not show any abnormal findings such as inflammation, fatty degeneration, or hepatocyte necrosis. No histopathological changes (e.g. glomerular or tubular damage) were observed in the liver tissues of the control group or the ASN pharmacopuncture group, and normal structures of the portal venule, bile duct, Kupffer cell, hepatic arteriole, and hepatocytes were observed. (Figs. 9 and 10).

Discussion

The characteristics of ASN in terms of oriental medicine, are sour, warm, and toxic. Therefore, the drug developed from ASN is used to treat stomachaches, palpitations, and lymphadenopathy. In oriental medicine, ASN acts in the blood to eliminate wind and dampness, stops pain through Qi, and relieves blood stasis. Consequently, they are effective for back, leg, and abdominal pain (especially the upper abdomen), gastric ulcers, headaches, and contusion injury.

Plants used in ASN pharmacopuncture have been traditionally used in China are mainly distributed from the Chinese provinces of Hebei, Shanxi, Southern Gansu, Sichuan, and Guizhou province. In modern times, it has been used to relieve arthralgia and lumbar

Table 7. The Hematological Values of Male Mice in the 4-week Toxicity Study of ASN Pharmacopuncture.

Index	Normal	ASN-L	ASN-M	ASN-H
White blood cell (10^3/μL)	3.16 ± 0.09	3.23 ± 0.08	3.11 ± 0.32	3.27 ± 0.27
Red blood cell (10^6/μL)	8.22 ± 0.85	8.37 ± 0.31	8.26 ± 0.18	8.58 ± 0.45
Hemoglobin (g/dL)	11.00 ± 0.78	10.91 ± 1.33	11.11 ± 0.68	10.97 ± 1.05
Hematocrit (%)	32.44 ± 2.15	30.97 ± 2.44	31.29 ± 2.11	31.94 ± 0.61
Platelet (10^3/μL)	945.41 ± 73.46	940.67 ± 52.67	948.51 ± 33.08	943.44 ± 56.29
Mean cell volume (fl)	41.83 ± 1.18	42.47 ± 3.87	41.43 ± 4.97	41.95 ± 1.34
Mean cell hemoglobin (pg)	12.78 ± 0.35	12.84 ± 2.37	12.74 ± 0.92	13.72 ± 1.70
Mean cell hemoglobin Concentration (g/dL)	29.70 ± 1.77	31.79 ± 3.12	31.25 ± 3.36	31.73 ± 3.47

The values represent mean ± SD.
Normal: Male BALB/c mice group injected with normal saline.
ASN-L: Male BALB/c mice group injected with low dose of ASN pharmacopuncture (15.63 mg/kg).
ASN-M: Male BALB/c mice group injected with intermediate dose of ASN pharmacopuncture (31.25 mg/kg).
ASN-H: Male BALB/c mice group injected with high dose of ASN pharmacopuncture (62.5 mg/kg).
ASN, Aconitum sinomontanum Nakai.

Table 8. The Hematological Values of Female Mice in the 4-week Toxicity Study of ASN Pharmacopuncture.

Index	Normal	ASN-L	ASN-M	ASN-H
White blood cell (10^3/μL)	3.29 ± 0.04	3.24 ± 0.25	3.19 ± 0.15	3.1 ± 0.45
Red blood cell (10^6/μL)	8.15 ± 0.25	8.14 ± 0.24	8.12 ± 0.65	8.2 ± 0.63
Hemoglobin (g/dL)	10.74 ± 0.87	10.37 ± 0.69	10.58 ± 1.06	10.52 ± 0.91
Hematocrit (%)	33.1 ± 0.54	31.97 ± 2.62	31.11 ± 1.49	31.76 ± 0.79
Platelet (10^3/μL)	937.76 ± 8.26	941.72 ± 84.83	939.82 ± 21.28	938.3 ± 49.73
Mean cell volume (fl)	40.74 ± 2.08	41.38 ± 1.27	39.83 ± 2.39	42.5 ± 2.83
Mean cell hemoglobin (pg)	13.79 ± 0.62	13.78 ± 1.54	12.92 ± 1.3	13.19 ± 0.84
Mean cell hemoglobin concentration (g/dL)	31.84 ± 1.73	31.41 ± 3.46	30.94 ± 0.43	31.92 ± 0.42

The values represent mean ± SD.
Normal: Female BALB/c mice group injected with normal saline.
ASN-L: Female BALB/c mice group injected with low dose of ASN pharmacopuncture (15.63 mg/kg).
ASN-M: Female BALB/c mice group injected with intermediate dose of ASN pharmacopuncture (31.25 mg/kg).
ASN-H: Female BALB/c mice group injected with high dose of ASN pharmacopuncture (62.5 mg/kg).
ASN, Aconitum sinomontanum Nakai.

Table 9. The Serum Biochemical Values of Male Mice in the 4-week Toxicity Study of ASN Pharmacopuncture.

Index	Normal	ASN-L	ASN-M	ASN-H
Total protein (g/dL)	4.99 ± 0.36	5.14 ± 0.17	4.97 ± 0.24	5.01 ± 0.17
Albumin (g/dL)	1.57 ± 0.09	1.58 ± 0.05	1.53 ± 0.08	1.52 ± 0.05
Total bilirubin (mg/dL)	0.12 ± 0.03	0.11 ± 0.04	0.10 ± 0.01	0.11 ± 0.02
Alkaline phosphatase (IU/L)	39.61 ± 5.19	40.28 ± 2.45	38.72 ± 7.19	39.32 ± 1.89
Aspartate aminotransferase (IU/L)	52.52 ± 2.56	49.19 ± 2.18	50.24 ± 2.11	50.31 ± 6
Alanine aminotransferase (IU/L)	18.77 ± 1.15	19.71 ± 3.03	21.51 ± 1.72	20.01 ± 2.29
Creatinine (mg/dL)	0.47 ± 0.1	0.45 ± 0.12	0.45 ± 0.08	0.46 ± 0.15
Blood urea nitrogen (mg/dL)	17.28 ± 1.57	18.12 ± 1.86	17.23 ± 1	17.59 ± 1.91
Cholesterol (mg/dL)	156.06 ± 16.1	155.85 ± 8.16	145.78 ± 10.23	153.91 ± 23.49
Triglyceride (mg/dL)	70.24 ± 5.11	69.44 ± 7.43	67.61 ± 4.03	67.75 ± 4.9

The values represent mean ± SD.
Normal: Male BALB/c mice group injected with normal saline.
ASN-L: Male BALB/c mice group injected with low dose of ASN pharmacopuncture (15.63 mg/kg).
ASN-M: Male BALB/c mice group injected with intermediate dose of ASN pharmacopuncture (31.25 mg/kg).
ASN-H: Male BALB/c mice group injected with high dose of ASN pharmacopuncture.(62.5 mg/kg).
ASN, Aconitum sinomontanum Nakai.

Table 10. The Serum Biochemical Values of Female Mice in the 4-week Toxicity Study of ASN Pharmacopuncture.

Index	Normal	ASN-L	ASN-M	ASN-H
Total protein (g/dL)	5.08 ± 0.39	5.14 ± 0.17	5.01 ± 0.32	5.04 ± 0.6
Albumin (g/dL)	1.49 ± 0.10	1.51 ± 0.05	1.58 ± 0.14	1.57 ± 0.08
Total bilirubin (mg/dL)	0.13 ± 0.05	0.12 ± 0.03	0.12 ± 0.04	0.12 ± 0.02
Alkaline phosphatase (IU/L)	39.27 ± 3.66	41.15 ± 4.37	41.92 ± 4.23	41.95 ± 2.77
Aspartate aminotransferase (IU/L)	49.35 ± 2.87	48.59 ± 4.98	50.07 ± 4.17	50.95 ± 5.4
Alanine aminotransferase (IU/L)	20.41 ± 2.59	19.53 ± 4.53	19.31 ± 3.22	19.3 ± 5.54
Creatinine (mg/dL)	0.50 ± 0.12	0.45 ± 0.06	0.51 ± 0.11	0.47 ± 0.05
Blood urea nitrogen (mg/dL)	16.77 ± 4.21	18.02 ± 1.54	16.64 ± 1.55	18.64 ± 1.30
Cholesterol (mg/dL)	153.49 ± 16.48	146.78 ± 6.88	152.35 ± 15.07	151.26 ± 15.77
Triglyceride (mg/dL)	68.67 ± 7.79	68.64 ± 7.02	69.51 ± 4.18	70.44 ± 8.66

The values represent mean ± SD.
Normal: Female BALB/c mice group injected with normal saline.
ASN-L: Female BALB/c mice group injected with low dose of ASN pharmacopuncture (15.63 mg/kg).
ASN-M: Female BALB/c mice group injected with intermediate dose of ASN pharmacopuncture (31.25 mg/kg).
ASN-H: Female BALB/c mice group injected with high dose of ASN pharmacopuncture (62.5 mg/kg).
ASN, Aconitum sinomontanum Nakai.

Table 11. The Relative Organ Weight Values of Male Mice in the 4-week Toxicity Study of ASN Pharmacopuncture.

Group	Normal	ASN-L	ASN-M	ASN-H
Heart	0.14 ± 0.02	0.12 ± 0.02	0.13 ± 0.01	0.13 ± 0.03
Liver	1.31 ± 0.07	1.33 ± 0.18	1.36 ± 0.12	1.31 ± 0.08
Kidney (right)	0.24 ± 0.04	0.26 ± 0.04	0.22 ± 0.04	0.23 ± 0.01
Kidney (left)	0.23 ± 0.02	0.25 ± 0.04	0.23 ± 0.02	0.22 ± 0.02
Brain	0.42 ± 0.02	0.46 ± 0.03	0.46 ± 0.03	0.43 ± 0.02
Lung	0.28 ± 0.02	0.29 ± 0.02	0.3 ± 0.03	0.27 ± 0.05
Spleen	0.13 ± 0.05	0.12 ± 0.02	0.12 ± 0.01	0.12 ± 0.02
Thymus	0.08 ± 0.03	0.09 ± 0.01	0.09 ± 0.01	0.11 ± 0.03
Testis	0.08 ± 0.01	0.1 ± 0.03	0.08 ± 0.02	0.08 ± 0.02
Gastrocnemius	0.14 ± 0.03	0.13 ± 0.04	0.13 ± 0.03	0.14 ± 0.03

The values represent mean ± SD.
Normal: Male BALB/c mice group injected with normal saline.
ASN-L: Male BALB/c mice group injected with low dose of ASN pharmacopuncture (15.63 mg/kg).
ASN-M: Male BALB/c mice group injected with intermediate dose of ASN pharmacopuncture (31.25 mg/kg).
ASN-H: Male BALB/c mice group injected with high dose of ASN pharmacopuncture (62.5 mg/kg).
ASN, Aconitum sinomontanum Nakai.

Table 12. The Relative Organ Weight Values of Female Mice in the 4-week Toxicity Study of ASN Pharmacopuncture.

Group	Normal	ASN-L	ASN-M	ASN-H
Heart	0.13 ± 0.02	0.13 ± 0.02	0.14 ± 0.02	0.12 ± 0.02
Liver	1.32 ± 0.07	1.32 ± 0.03	1.33 ± 0.07	1.35 ± 0.05
Kidney (right)	0.24 ± 0.03	0.26 ± 0.03	0.25 ± 0.02	0.24 ± 0.05
Kidney (left)	0.23 ± 0.03	0.23 ± 0.01	0.22 ± 0.02	0.24 ± 0.02
Brain	0.46 ± 0.04	0.44 ± 0.02	0.44 ± 0.06	0.44 ± 0.05
Lung	0.26 ± 0.02	0.25 ± 0.03	0.24 ± 0.03	0.26 ± 0.03
Spleen	0.14 ± 0.04	0.12 ± 0.02	0.14 ± 0.02	0.14 ± 0.01
Thymus	0.10 ± 0.02	0.1 ± 0.02	0.12 ± 0.05	0.10 ± 0.02
Testis	0.09 ± 0.02	0.09 ± 0.02	0.08 ± 0.02	0.08 ± 0.02
Gastrocnemius	0.14 ± 0.02	0.12 ± 0.04	0.12 ± 0.04	0.13 ± 0.02

The values represent mean ± SD.
Normal: Female BALB/c mice group injected with normal saline.
ASN-L: Female BALB/c mice group injected with low dose of ASN pharmacopuncture (15.63 mg/kg).
ASN-M: Female BALB/c mice group injected with intermediate dose of ASN pharmacopuncture (31.25 mg/kg).
ASN-H: Female BALB/c mice group injected with high dose of ASN pharmacopuncture (62.5 mg/kg).
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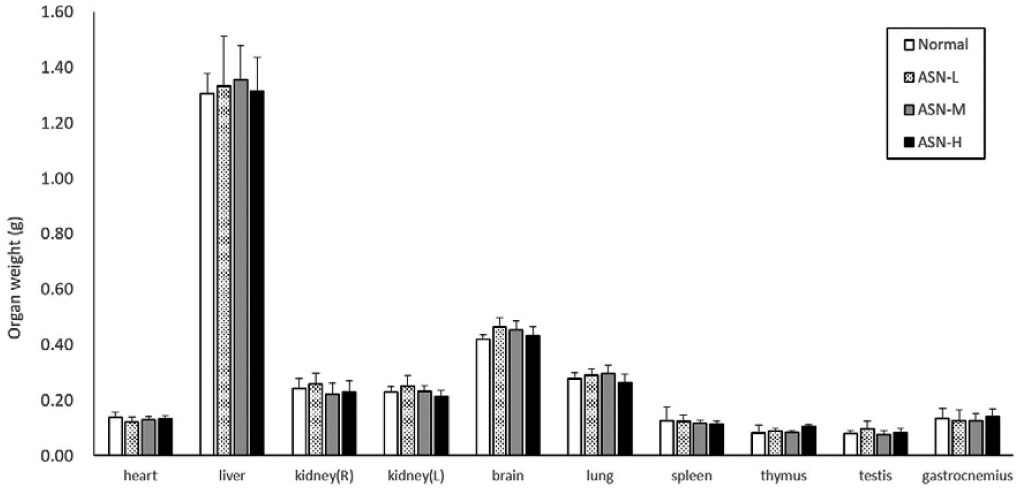

Fig. 7. The relative organ weight values of male mice in the 4-week toxicity study of ASN pharmacopuncture.
The values represent mean ± SD.
Normal: Male BALB/c mice group injected with normal saline.
ASN-L: Male BALB/c mice group injected with low dose of ASN pharmacopuncture (15.63 mg/kg).
ASN-M: Male BALB/c mice group injected with intermediate dose of ASN pharmacopuncture (31.25 mg/kg).
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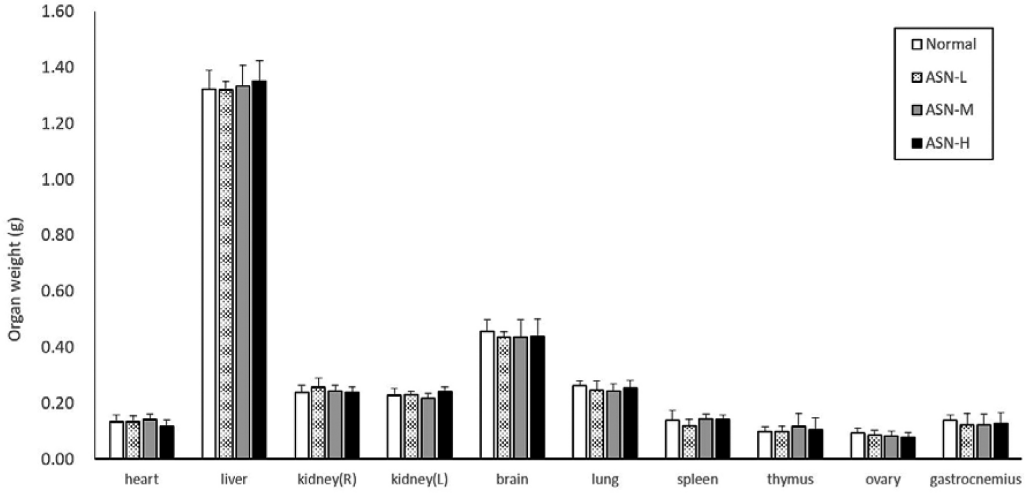

Fig. 8. The relative organ weight values of female mice in the 4-week toxicity study of ASN pharmacopuncture.
The values represent mean ± SD.
Normal: Female BALB/c mice group injected with normal saline.
ASN-L: Female BALB/c mice group injected with low dose of ASN pharmacopuncture (15.63 mg/kg).
ASN-M: Female BALB/c mice group injected with intermediate dose of ASN pharmacopuncture (31.25 mg/kg).
ASN-H: Female BALB/c mice group injected with high dose of ASN pharmacopuncture (62.5 mg/kg).

pain in patients with rheumatoid arthritis. A recent study reported that ASN inhibits fibroblast-like synoviocytes, which play an important role in the development of rheumatoid arthritis [6]. In Korea, it has been reported to have analgesic properties against thermal, chemical, and physical pain [5]. As for lappaconitine, a major component of ASN, various studies have reported its anti-inflammatory and analgesic effects, such as in burns patients, and patients with postoperative and chronic pain [7-10].

ASN pharmacopuncture used for pain control in various diseases requires ASN toxicity studies before clinical trials. According to the guidelines for non-clinical testing of herbal medicines by the Korea Food and Drug Administration, toxicity tests of herbal medicine are classified as a single-use, repeated administration, genetic, reproductive toxicity tests, and carcinogenicity tests [11]. Single-dose and repeated dose toxicity tests in animals are usually performed in rats or mice [12].

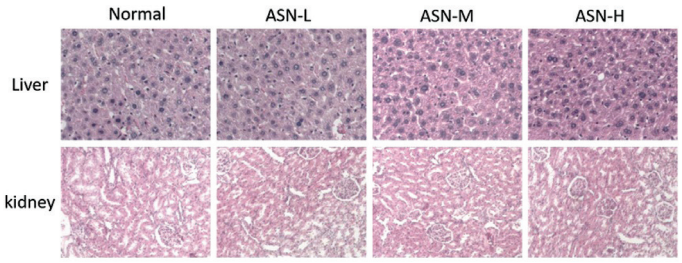

Fig. 9. Hematoxylin-eosin stained liver and kidney tissues from male mice injected with ASN pharmacopuncture for 4 weeks.
Normal: Male BALB/c mice group injected with normal saline.
ASN-L: Male BALB/c mice group injected with low dose of ASN pharmacopuncture (15.63 mg/kg).
ASN-M: Male BALB/c mice group injected with intermediate dose of ASN pharmacopuncture (31.25 mg/kg).
ASN-H: Male BALB/c mice group injected with high dose of ASN pharmacopuncture (62.5 mg/kg).

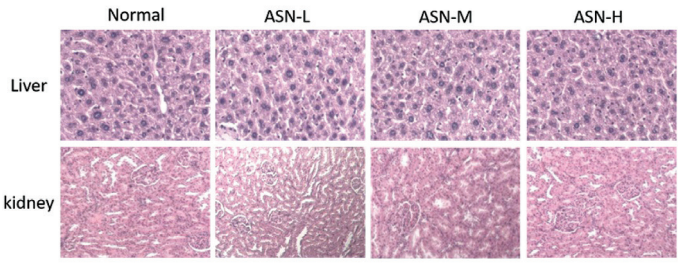

Fig. 10. Hematoxylin-eosin stained liver and kidney tissues from female mice injected with ASN pharmacopuncture for 4 weeks.
Normal: Female BALB/c mice group injected with normal saline.
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No-Observed-Effect-Level is a term for the maximum unaffected capacity without causing toxic and pharmacological changes; No-Observed-Adverse-Effect-Level (NOAEL) indicates the maximum non-toxic capacity that causes no adverse effects or is clearly not associated with the disease. Lowest-Observed-Adverse-Effect-Level, defines the minimum toxic dose that causes side effects [13]. To measure these indicators NOAEL, and the Lowest-Observed-Adverse-Effect-Level are estimated based on results of the single-dose and repeated administration toxicity tests at 2 to 4 weeks, and 1, 3, and 6 months or more, and by classifying study groups into at least 3 stages during toxicological testing. In this current study both single and 4-week repeated toxicity tests were performed using BALB/c mice to investigate the toxicity and safety of ASN pharmacopuncture and NOAEL. LD50 is the dose that causes death in 50 % of the experimental animals. The concept of LD50 was first developed by Trevan [14] around 1927, and it is one of the criteria for assessing toxicity of the test material in the shortest amount of time [15]. It is also used as a basis for setting dose planning in future toxicity tests [16].

In this study, the dose for the intermediate concentration for the single-dose toxicity test, and the high concentration for the 4-week repeated toxicity tests (equivalent to a human adult weighing 60 kg), was determined. Partial skin rashes were reported in rats treated with 500 mg/kg ASN [6], therefore, the single-dose toxicity studies were selected as 31.25 mg/kg (low), 62.5 mg/kg (intermediate), and 125 mg/kg (high) concentration groups. For the 4-week repeated toxicity test, the ASN concentrations were set at 15.625 mg/kg/day (low), 31.25 mg/kg/day (intermediate), and 62.5 mg/kg/day (high) concentration groups. The results demonstrated that neither weight gain, morbidities or mortalities were significantly different between groups. In addition, the results of blood hematological and biochemical tests showed no significant change in either the ASN pharmacopuncture groups or the control group after repeated treatment with ASN pharmacopuncture for single- and 4 week experiments. In conclusion, ASN pharmacopuncture treatment at 125 mg/kg in BALB/c mice after single- or 4-week repeated toxicology studies did not show any abnormal symptoms, weight loss, or mortality; and there were no significant changes in hematological and biochemical indicators.

Since most toxic substances that enter the body undergo hepatic metabolism after absorption, the liver is likely to contain metabolites of several compounds, and there is a high possibility of perihepatic cellular damage when toxic substances are processed [17,18]. In this study, the liver was observe for changes that may have occurred with repeated administration of ASN pharmacopuncture for 4 weeks. Investigation of the livers of the BALB/c mice (by measuring organ weights and checking liver function tests including total protein, albumin, total bilirubin, alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase [19-21] after 4 weeks of repeated toxicity tests were performed. Histopathological evaluations of the liver tissues (using H&E stain) were also performed to identify changes in liver tissue. The results showed no significant changes in liver function tests, liver weight, and histopathological results in both single and 4-week repeated groups after 4 weeks of repeated administration.

The kidney filters 20 % of the cardiac output [22] and excretes toxic substances from the body [23]. It is more sensitive to toxic metabolites than any other organ because of its function and the concentration of blood that it processes [17,18]. In this study, changes in the kidneys after repeated ASN pharmacopuncture administration for 4 weeks were assessed. Blood biochemical indicators were evaluated [24,25] for the 4-week repeated toxicity test, weight, tissue pathology were examined (using H&E stain) to identify minor changes in kidney tissue. The results showed no significant changes in the biochemical indicators related to renal function, gross and histopathological examination, and organ weight after 4 weeks of repeated administration. In summary, 4 weeks of ASN pharmacopuncture treatment did not cause toxicity to the liver and kidneys of the mice.

In addition, the weight of the heart, liver, brain, lungs, spleen, thymus, reproductive organs, and skeletal muscles located close to the site of ASN pharmacopuncture injection showed no significant changes during the treatment period in both ASN pharmacopuncture and control groups.

ASN pharmacopuncture treatment in BALB/c mice in single- and 4-week repeated doses toxicity tests in this study were safe. The NOAEL for single administration of ASN pharmacopuncture was more than 125 mg/kg, whilst 4 weeks of administration the NOAEL was more than 62.5 mg/kg/day in BALB/c mice.

There were some limitations to this study. Toxicity tests of ASN

were conducted for 4 weeks, but longer-term studies are needed to safely recommend use of ASN pharmacopuncture. In addition, further studies such as genotoxicity, reproductive, carcinogenicity, and other toxicity tests associated with ASN pharmacopuncture treatment are needed. Moreover, the study observed toxicity in a single species, so future studies in other species are recommended. In addition, further studies on the side effects and mortality are required for concentrations of 500 mg/kg or higher when skin rashes may occur. Stability studies of active ingredients to be used for pharmacopuncture are warranted.

Conflicts of Interest

The authors declare that there are no conflicts of interest

References

[1] Korean Pharmacopuncture Institute. Pharmacopuncturology. Seoul (Korea); Elsevier Korea; 2008 p. 3-8. [in Korean].

[2] Ren YS, Zhang P, Yan D, Wang JB, Du XX, Xiao XH. A Strategy for The Detention of Quality Fluctuation of A Chinese Herbal Injection Based on Chemical Fingerprinting Combined with Biological Fingerprinting. J Pharm Biomed Anal 2011;56:436-442.

[3] Gao BZ. New Chinese Medicine Dictionary. middle Taibei (Taiwan); Xinwenli publishing company; 1982. 273 p. [in Chinese].

[4] Hwa EU, Ryu SB, Yang JG, Yang HK, Yang CH, Ung SW et al. Junghwaboncho. Shanghai (China); Sanghai gichul publisher; 1999. p. 1746-1747. [in Chinese].

[5] Lee JH[Dissertation]. The analgesic effect and safety of Aconitum sinomont anumNakaipharmacopuncture in Sprague-Dawley rats. Gyungsan (Korea): Daegu Haany University; 2019. p. 1-64. [in Korean].

[6] Deng X, Zheng LP, Mu ZQ, Lai R, Niu GP, Tu LP et al. The inhibitory effect of Aconiti Sinomontani Radix extracts on the proliferation and migration of human synovial fibroblast cell line SW982. J Ethnopharmacol 2018; 213:321-327.

[7] Wang YZ, Xiao YQ, Zhang C, Sun XM. Study of analgesic and anti-inflammatory effects of lappaconitine gelata. J Tradit Chin Med 2009;29:141-145.

[8] Sun ML, Ao JP, Wang YR, Huang Q, Li TF, Li XY et al. Lappaconitine, a C18-diterpenoid alkaloid, exhibits antihypersensitivity in chronic pain through stimulation of spinal dynorphin A expression. Psychopharmacology(Berl) 2018;235:2559-2571.

[9] Yang CL, Wei ZR, Zhang TH, Zeng XQ, Wu BH. Effects of lappaconitine on pain and inflammatory response of severely burned rats and the mechanism. Zhonghua Shao Shang Za Zhi 2017;33:374-380. [in Chinese].

[10] Gong QA, Li M. Effect of Lappaconitine on Postoperative Pain and Serum Complement 3 and 4 Levels of Cancer Patients Undergoing Rectum Surgery. Zhongguo Zhong Xi Yi Jie He Za Zhi 2015;35:668-672. [in Chinese].

[11] National Institute of Food and Drug Safety Evaluation [Internet]. Guideline on Non-Clinical Documentation for Herbal Medicinal Product. [cited 2017 Oct 1]. Available from: https://www.nifds.go.kr/brd/m_15/list.do. [in Korean].

[12] Ministry of Food & Drug Safety [Internet]. A Flow Chart on Toxicity Test of Medicine (2015). [cited 2015 Nov 11]. Available from: http://www.mfds.go.kr/index.do?mid=1013&seq=10128&cmd=v. [in Korean].

[13] Park YC, Cho MH. A New Way in Deciding NOAEL Based on The Findings From GLP-Toxicity Test. Toxicol Res 2011;27:133-135.

[14] Trevan JW. The Error of Determination of Toxicity. Proc R Soc Lond B Biol Sci 1927;101:483-514.

[15] Choi SY. Toxicology. Ulsan (Korea); Ulsan University publishing department: 1987. p. 189-207, 298-299. [in Korean].

[16] Kim SK, Kim KC, Lee BM. Mathematical and Statistical Characterization of LD (50) Estimation. Toxicol Res 2014;20:321-324.

[17] Jung SY, kim GY, Kim BH, Jo YB. Toxicology. Seoul (Korea): Donghwa technology; 2000. p.15-21, 118-194. [in Korean].

[18] Ko YS, Yun SH, Kim MK, Song YS, Shin BC. Literatural Study on Toxicity of Herbs used in <Bangyakhappyeon>. J Physiol Pathol Korean Med 2005;19:1120-1128. [in Korean].

[19] Lee YJ, Hwang WD, Lee JW, Jeong HJ, Bae SH, Kim KG. The Study of Safety of Herbal Medicine Including Fuzi on High Range of LFT, RFT Patients. Herb Formula Sci 2011;19:179-189. [in Korean].

[20] Park YC, Park HM, Lee SD. Inducible Mechanisms for Hepatotoxicity Caused by Traditional Korean Medicines in a View of Toxicology. J Korean Orient Med 2011;32:48-67. [in Korean].

[21] Pessayre D, Larrey D, Biour M. Drug-induced liver injury. Oxford (UK): Oxford University Press; 1999. p. 1261-1315.

[22] Jeongdam editorial department. Step to Internal Medicine part 5 Kidney (2018). Seoul (Korea): Jeongdam; 2016. 4 p. [in Korean].

[23] Kim SY, Mun AR. Drug-Induced Nephrotoxicity and Its Biomarkers. Biomol Ther (Seoul) 2012;20:268-272.

[24] Kim EJ, Kan DG, Lee AS, Choi DH, Cho KH, Kim SY et al. Study on Kidney Toxicity of BDR-29 for Treatment Vascular Diseases in Rat. Herb Formula Sci 2008;16:163-169. [in Korean].

[25] Kim DW. The Change of Renal Function in Patients with Long Term Herb Medication by Frequently Prescribed Formular. J Physiol Pathol Korean Med 2004;18:1207-1212.