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Original Article

Four-week Repeated Dose Toxicity Test for Myelophil in SD Rats

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Aim : To evaluate the pharmaceutical safety of the herbal formula Myelophil, composed of Astragali Radix and Salviae Radix, via systemic subacute toxicological study using SD rats.
Methods : Forty male and 40 female SD rats were fed with Myelophil (5000, 2500 or 1250 mg/10 mL/kg) or distilled water for four weeks. Adverse effects were examined intensively by comparing the differences between normal and drug-administered groups using clinical signs, necropsies, histopathologic findings, hematology, urinalysis, and blood biochemical analysis.
Results : No altered clinical symptoms including body weight, diarrhea, anorexia, death, and abnormal necropsy of major organs were observed in male or female rats. No drug-induced abnormalities in histopathological finding, hematological values, urinalysis, and blood biochemical values were found at any doses of Myelophil.
Conclusion : Myelophil should be very safe when used in a clinical application with a wide therapeutic index.

Key Words : Myelophil, Astragali Radix, Salviae Radix, herbal remedy, safety, toxicological study

Introduction

A variety of medicinal plants and botanical drugs have been widely used all over the world, as primary therapeutics or supplements for treating various diseases[1,2]. These products are generally regarded as non-toxic due to their natural origin and long history of being used without serious adverse reactions. However, many current medical issues are related to a lack of scientific evidence about the efficacy, quality control and especially safety of herbal remedies[3,4]. Particularly, plenty warnings have been reported about the potential hepatotoxicity of herbal products[5,6].

Myelophil is a mixture of equal volumes of Astragali Radix and Salviae Radix extracts. This drug was developed for use with cancer patients with chemo/radio-therapy-induced side effects. Its pharmaceutical properties are known to reduce bone marrow suppression by chemotherapy in animal study, and to improve quality of life of human subjects with chronic fatigue symptoms[7,8]. These two medicinal plants are believed to have no toxicity, so Myelophil is strongly assumed to be very safe. However, its safety had not been verified via scientifically-designed protocol.

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In the present study, to examine the toxicity of Myelophil, and to estimate no observable adverse effect level (NOAEL), a four-week repeated dose oral toxicity study was conducted in SD rats. This study will help to build a basis of scientific data regarding the safety of herbal plant-derived remedies in the field of traditional Korean medicine.

Materials and method

1. Myelophil and fingerprinting using HPTLC

Myelophil (containing 50% water) was prepared by Samik Pharmaceutical Company (Seoul, Korea) as follows. Briefly, the mixture of Astragali Radix (VS NO: AM-2006-02-ra) and Salviae mitiorrhizae Radix (VS NO: SM-2006-01-Ra) (1:1) was extracted for 20 h at 80℃ according to over-the-counter Korean monographs. A final product with 20.52% (w/w) yield was stored for future use (VS NO: MP-2006-01-WE). To get information of components contained within Myelophil, high performance thin layer chromatography (HPTLC) was performed using its two major components (water extracts of Astragali Radix and Salviae Radix) with two reference compounds, rosmarinic acid and formononetin respectively (Fig. 1).

2. Animals and experimental design

SD rats were purchased from Orientbio (Gapyeonggun, Gyeonggido, Korea). All animals were acclimated and quarantined for 1 week and healthy animals were selected to use in the experiment. Specific pathogen-free SD rats were housed in an environmentally controlled room with temperature of 20.1~23.1℃, relative humidity of 45.2~68.3%, air ventilation of 10~15 air changes/h, a 12 h light/dark cycle of 200~300 lux, and feed and water available *ad libitum*. During the whole experimental period, temperature and relative humidity were recorded automatically every 30 min, and circumstance parameters such as intensity of illumination were checked regularly. There was no change to affect the experiment. Rodent feed sterilized with radiation (2.0 Mrad) was obtained from Purina Korea (Gyeonggido, Korea) and UV sterilized water was used. Feed and water were tested for contamination and there was nothing to affect the experiment.

Forty male and 40 female rats were assigned into four groups. The rats were administrated with Myelophil (5000, 2500 or 1250 mg/10 mL/kg) or distilled water once a day for 4 weeks. Mortality and clinical signs such as appearance, autonomic nerve and so on were observed every day. Body weight and food/water consumption were determined once a week. Urine test and ophthalmological test were performed in the last week. This experiment was performed in safety assessment center of Korea Testing and Research Institute (Gimpo, Korea).

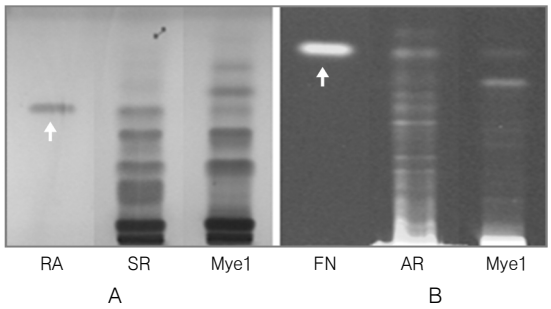

Fig. 1. HPTLC-based fingerprint.

HPTLC analysis was performed to characterize Myelophil using two known components. Rosmarinic acid (RA) for Salviae Radix (SR) and formononetin (FN) for Astragali Radix (AR) were subjected to HPTLC. The separated components were visualized in white light after derivatization (A) or under UV 366 nm (B).

3. Hematological and blood biochemical analysis and urinalysis

At the end of experiment, the animals were fasted overnight and blood was collected from the abdominal artery under anesthesia with ether. Two milliliters of blood was transferred into a CBC bottle (EDTA 3K, Sewon medical, Busan, Korea) and analyzed using an autohematology analyzer (ADVIA120E, Bayer, NY, USA). For blood biochemical analysis, blood was transferred into a Vacutainer (Sodium citrate 3.2%, BD, NJ, USA) and centrifuged at 3000 rpm for 10 min. The plasma was isolated and used to determine aggregation time using a coagulometer (Coagrex-100s, International reagent cooperation, Hyogo, Japan). Blood was stood to aggregation for 1 h at room temperature and centrifuged at 3000 rpm for 10 min. Serum were isolated and used to biochemical analysis using automatic serum analyzer (Hitachi 7060, Tokyo, Japan) and Na/K/Cl analyzer (Bayer 644, PA, USA).

Urinalysis was performed before the last day of the experiment. The specific gravity, pH, leukocyte, nitrite, protein, glucose, ketone, urobilinogen, bilirubin, and blood were analyzed using Multistix 10SG strip (Bayer, IND, USA) and urine analyzer (Clinitek 500, MN, USA).

4. Histopathological examination

After bleeding of residual blood, internal organs were weighed and gross findings were recorded. All organs including heart, liver kidney, adrenal, spleen, testes (uterus), epididymis, prostate, brain, thymus, thyroid, pituitary gland, and lung were weighed and fixed with 10% neutral formalin individually, but the testes and epididymis were fixed with Bouin's solution individually for histopathological examination.

5. Statistical analysis

Body weight, feed and water consumption, hematological data, blood biochemical data and organ weight were analyzed for homogeneity of variance using Levene's test. Tests of significance were performed using ANOVA analysis for homogeneous data and these were reinspected with Scheffe's test using SPSS program (Ver 10.1).

Results

1. Clinical signs and body and organ weights

No dead animals were observed in any groups during the experimental period. Two female rats in the low and high dosage group were shown to have alopecia. All groups gained body weight during the

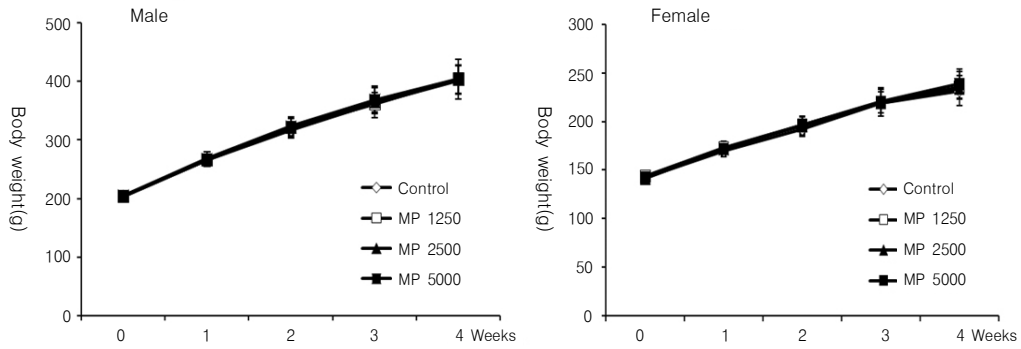

Fig. 2. Change of body weight.

The rats were administered Myelophil (5000, 2500, 1250 mg/10 mL/kg) or distilled water once a day for 4 weeks. Body weight was measured every week. Values represent the mean ± SD.

experimental period, but these changes of body weight were not significant compared to the control group (Fig. 2). Major organs of all animals were inspected by naked eye and no abnormal findings were observed. In ophthalmological test, no abnormal finding was observed in any group. Particular change in food and water consumption was not observed.

2. Hematological and blood biochemical values

Administration of Myelophil didn't affect hematological parameters such as cell counts of red blood cells, white blood cells or platelets. No difference was shown in prothrombin time and activated partial thromboplastin time among the four groups (Table 1).

On the other hand, some changed serum parameters in the Myelophil-administrated group were shown (Table 2). In the male rats, triglycerides were significantly increased in the low dosage group. In the female rats of high dosage, the levels of urea nitrogen and glucose significantly increased.

Significant change was not observed in urinalysis (data not shown).

3. Histopathological findings

Diffuse micro lipid droplet deposit and focal lymphocyte aggregation were observed in livers of female and male administered groups as well as control. In kidney of the male control and treatment groups, two cases of focal lymphocyte aggregation,

Table 1. Hematological Values in Male and Female Rats

		Male (10 rats per group)				Female (10 rats per group)			
		Control	MP 1250	MP 2500	MP 5000	Control	MP 1250	MP 2500	MP 5000
WBC (k/μL)		9.65±2.39	9.56±1.77	10.3±2.93	8.94±2.56	6.56±2.14	7.01±3.5	5.97±1.99	6.31±1.33
WBC differential counting (k/μl)	NE	1.87±0.41	1.71±0.38	1.81±0.61	1.69±0.72	0.82±0.26	1.22±0.54	0.89±0.56	1.11±0.66
	LY	7.35±2.25	7.42±1.64	8.13±2.42	6.88±1.85	5.46±2.25	5.45±2.92	4.77±1.76	4.96±0.81
	MO	0.29±0.07	0.28±0.11	0.24±0.08	0.24±0.13	0.15±0.05	0.17±0.1	0.2±0.11	0.13±0.03
	EO	0.09±0.03	0.11±0.04	0.08±0.01	0.09±0.04	0.09±0.04	0.13±0.15	0.09±0.04	0.08±0.02
	BA	0.02±0.01	0.03±0.01	0.03±0.02	0.02±0.01	0.02±0.01	0.02±0.02	0.02±0.01	0.01±0.01
RBC (M/μL)		7.66±0.19	7.79±0.26	7.73±0.26	7.66±0.28	7.64±0.29	7.72±0.22	7.54±0.47	7.59±0.11
Hemoglobin (g/dL)		15.6±0.4	15.6±0.6	15.6±0.5	15.7±0.6	15.4±0.4	15.1±0.6	15±0.6	15±0.5
HCT (%)		41.8±1.3	41.8±1.4	41.8±1.4	41.7±1.9	40.6±1.1	40.1±1.2	39.7±1.6	39.7±1.1
MCV (fL)		54.5±1.4	53.8±2	54.1±1.4	54.5±2	53.1±1.3	52±1.4	52.7±1.8	52.4±1.4
MCH (pg)		20.4±0.5	20±0.7	20.2±0.5	20.4±0.7	20.2±0.5	19.6±0.7	19.9±0.7	19.7±0.5
MCHC (g/dL)		37.4±0.3	37.3±0.4	37.3±0.4	37.5±0.4	38±0.3	37.7±0.7	37.8±0.6	37.7±0.6
Reticulocyte (%)		2.71±0.31	2.69±0.19	2.68±0.51	2.8±0.24	2.33±0.5	2.14±0.35	2.05±0.39	2.32±0.41
Platelet (k/μl)		1117±94	1117±76	1115±82	1159±79	1150±203	1199±111	1253±97	1173±104
PT (sec)		27.8±11.3	29.2±15.3	25±2.8	39.7±16.7	22.8±7	19.3±1.5	20.4±4.4	29.6±12.5
APTT (sec)		43.1±12.7	49.1±13.6	42.6±12	40.7±8.9	34.3±6.2	34.2±5	34.2±4.9	34±4.2

The rats were administered Myelophil (5000, 2500, 1250 mg/10 mL/kg) or distilled water once a day for 4 weeks. On the last day, blood was collected from the abdominal artery under anesthesia with ether, then, complete blood count was analyzed using autohemato analyzer.
MP: Myelophil; MCV: mean corpuscular volume; MCH: Mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; PT: prothrombin time; APPT: activated partial thromboplastin time. Data are expressed as mean ± SD.

Table 2. Blood Biochemical Values in Male and Female Rats

	Male (10 rats per group)				Female (10 rats per group)			
	Control	MP 1250	MP 2500	MP 5000	Control	MP 1250	MP 2500	MP 5000
T. protein (g/dL)	5.7±0.2	5.9±0.2	6.0±0.2	5.9±0.2	6.3±0.2	6.3±0.5	6.7±0.3	6.6±0.4
Albumin (g/dL)	2.4±0.1	2.4±0.1	2.5±0.1	2.5±0.1	2.8±0.2	2.8±0.3	2.9±0.2	2.9±0.3
T. bilirubin (mg/dL)	0.04±0.01	0.03±0.01	0.04±0.02	0.03±0.02	0.04±0.01	0.05±0.01	0.05±0.02	0.04±0.01
ALP (U/L)	451±66	423±86	466±72	413±57	296±59	297±94	279±71	303±85
AST (U/L)	89±14	83±15	90±12	97±19	117±27	95±20	102±15	98±13
ALT (U/L)	33±6	34±4	37±7	35±5	25±4	24±4	24±3	25±4
Creatinine (mg/dL)	0.5±0.0	0.5±0.0	0.5±0.1	0.5±0.1	0.5±0.1	0.5±0.1	0.6±0.1	0.5±0.0
BUN (mg/dL)	11.8±1.3	12±1.9	11.9±1.8	12.2±1.9	15.8±2.3	15.9±1.6	16.8±2.9	21.5±5.9*
Cholesterol (mg/dL)	64±12	71±11	68±8	73±10	74±12	69±17	77±16	80±16
Triglyceride mg/dL)	67±20	123±44*	93±38	89±36	16±9	16±8	19±10	21±7
Glucose (mg/dL)	170±17	164±15	165±18	166±20	143±18	150±25	137±14	181±22*
Na (mmol/L)	141±1	144±1	143±2	143±1	141±1	141±2	143±1	144±2
K (mmol/L)	3.98±0.24	4±0.17	4.14±0.32	3.97±0.29	4.42±0.30	4.29±0.40	4.39±0.41	4.36±0.18
Cl (mmol/L)	108±2	109±1	109±1	111±1	111±1	112±2	110±1	111±1

After administration of Myelophil (5000, 2500, 1250 mg/10 mL/kg) for 4 weeks, blood biochemistry was analysed. ALP: alkaline phosphatase; AST: aspartate aminotransferase; ALT: alanine aminotransferase; BUN: blood urea nitrogen. Significant difference was compared to values of control group with Scheffe's test (*, $P < 0.05$). Data are expressed as mean ± SD.

and focal tubular mineralization were observed. In the thyroid gland of the male treatment group, minimal focal lymphocyte infiltration was observed. However, there were no considerable changes between control and treatment groups (Fig. 3).

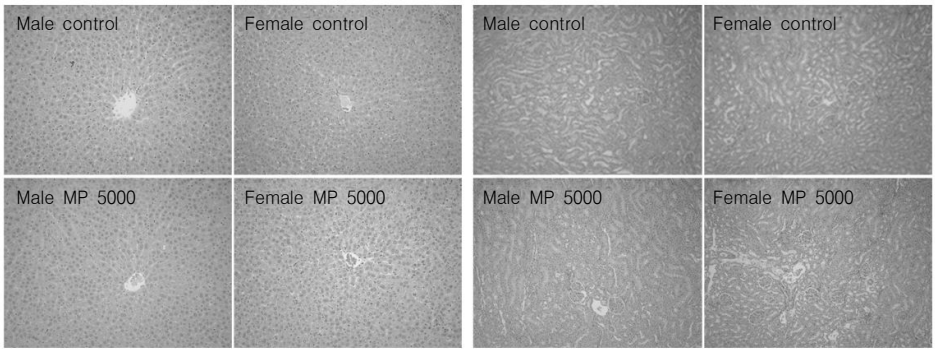

Fig. 3. Histopathological findings of liver and kidney.

Liver and kidney tissue at the highest dose of Myelophil and control group were fixed in 10% neutral formalin. Paraplast-embedded liver was sectioned (4 μm thick), and sections were stained with hematoxylin and eosin. The tissue slides were photographed at 200× magnification.

Discussion

Myelophil is an aqueous extract of Astragali Radix and Salviae Radix, which was developed for patients complaining of fatigue symptom or anti-cancer drug side effects such as suppression of bone marrow function[7,8]. Astragali Radix has been used in oriental clinics as an enhancer of *Qi*, and was known to have properties of immunomodulation, anti-aging, and anti-tumour[9-11]. Salviae Radix is a representative plant to improve blood-related disorders, with experimental evidence for haematopoietic, anti-oxidant, anti-hypertensive, and anti-inflammatory effects[12-15].

These two medicinal plants are believed to harbour no toxicity; however, no animal-based high-dose toxicological studies had been performed in conjunction with tests to confirm the drug's safety and efficacy in the appropriate dosages. Therefore, we herein first did the subacute toxicological tests to evaluate the wide-range tolerance and safety of Myelophil.

Male and female SD rats were orally administrated with various concentrations of Myelophil (5000, 2500 or 1250 mg/kg/day) for 4 weeks. The therapeutic dosage of Myelophil is about 100 mg per kg for one day; accordingly, this dosage is representative of over ten to fifty times of optimal human clinical dose.

As shown in the results above, there were no animal deaths throughout the experimental period. Alopecia appeared in only two rats in the female groups, but not in the other 78 rats observed. So, alopecia might be caused by wire cages. Other abnormal clinical signs besides alopecia were not shown. In body weight, food/water consumption, urine analysis, necropsy findings, hematological analysis, ophthalmological test, and histopathological examination, no changes associated with Myelophil administration were observed. Typical adverse signs of any drug include changes in appetite or feces; even a 50-fold dose of Myelophil did not affect appetite or feces in any group, nor did it affect body weight changes in any group.

In complete blood cell analysis, no change was observed among the four groups. The serum level of triglycerides revealed significant increase, it is doubtful since the results exhibited only in one low concentration in male. In the high dosage female group, urea nitrogen and glucose were significantly increased. However, the significance of these changes is not thought to be caused by Myelophil because the results did not repeated in the other sex group and were not correlated with histopathological finding or urinalysis.

To examine potential hepatotoxicity is very important in herbal products because all drugs or toxic agents are biologically processed primarily in the liver[16]. Serum values of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and bilirubin are among the most sensitive markers of liver damage[17]. As expected, treatment with Myelophil didn't affect serum levels of them compared to the control group.

So far, only one study partially presented the safety of Astragali Radix[17]. Moreover, there was a lack of toxicological data about not only Salviae Radix or Astragali Radix but also synergistic adverse effects when both drugs were combined. The present study confirmed that daily administration of Myelophil for 4 weeks at 5000, 2500 and 1250 mg/kg/day to SD rats has no adverse effects on clinical signs as well as blood/histopathology-based studies.

Taken together, it can be concluded that Myelophil could be safely used in a clinical application with a very large therapeutic index, and its NOAEL is assumed as to be over 5000 mg/kg per day. Also, this study added the scientific data supporting safety of herbal-derived remedies.

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