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녹용대보탕 열수 추출물의 실험적으로 유발된 랫트 아급성 출혈성 빈혈에 대한 효과

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ABSTRACT

Ameliorating Effects of *Nokyongdaebo-tang* on Experimental Subacute Hemorrhagic Anemia in Rats

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Objectives: The object of this study is to observe the possible ameliorating effects of *Nokyongdaebo-tang* (NYDBT) on the experimental subacute hemorrhagic anemia (SHA) in rats.

Methods: In the present study, SHA in rats was induced by exsanguinations from orbital plexus, and ameliorating effects of NYDBT was observed based on the changes of body and hematopoietic organ (spleen, liver and femur) weights, red blood cell (RBC) related hematological values, smear cytology, histopathological changes and immunohistochemistrical analysis of hematopoietic stem cells in the femur bone marrow, liver and spleen. In addition, the gastrointestinal motility and the surface mucosa thicknesses of remnant fecal pellets in the colon lumen, mucosa thicknesses and the mucous producing cell numbers in the colonic mucosa were analyzed to observe the digestive disorders, especially on the constipation, the major discomfort problems in iron supplement.

Results: SHA related abnormal anemic signs were markedly and dose-dependently inhibited by oral administration of NYDBT 500, 250 and 125 mg/kg in a condition of this experiment. In addition, no meaningful changes on the gastrointestinal motilities and mucous component on the colon and remnant feces were noticed in all three different dosages of NYDBT treated rats as compared with intact vehicle and SHA control rats in this study.

Conclusions: It, therefore, is expected that NYDBT will be promising as a novel alternative hematopoietic and therapeutic agent for anemia.

Key Words: *Nokyongdaebo-tang*, Ferromax, Subacute Hemorrhagic Anemia, Hematopoietic Effect

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I. Introduction

Anemia is a decrease in number of red blood cells (RBCs, erythrocytes) or less than the normal quantity of hemoglobin in the blood[1]. Acute blood loss anemia, also called hemorrhagic anemia, is specific type of anemia that sufficient decrease in RBC due to acute hemorrhage[1]. Although hemorrhagic shock was induced in a case of acute blood losses, over 20% in mammalian body, chronic or subacute blood losses can be induced the regenerative and iron deficient anemia[2].

Until now, hemorrhage related regenerative iron deficient anemia has been treated by iron supplements and they have been showed potent anti-anemic effects. However, they induced various side effects including digestive discomfort, especially constipation[4].

Therefore, new strategies highlighted as a modern concern to search hematopoietic potent anti-anemic agents have less toxic effects rather than simple alternative iron supplements, especially concerned on natural herbs or their mixtures[5].

As traditional medicine, anemia is belong to blood deficiency[6]. Relationship of qi and blood is explained as 'Qi can make blood, qi can move blood[7]'. Therefore, supplement with qi and blood is treatment as traditional medicine for anemia[7].

Nokyongdaebo-tang[8] (NYDBT) is a traditional medicine and aqueous polyherbal formula that has been used for several hundred years, predominantly to tonic purpose in a weak constitution and supplement with qi and blood[8] (Table 1). Studies have been reported the specific bioactivities of NYDBT, which include neuroprotective activities to Alzheimer's disease model[9], hypolipidemic effects[10], antithrombotic activities[11], anti-oxidative effects[12] and anti-aging activities[13]. However, there are no reports dealing anti-anemic activities of NYDBT, upon our knowledge, especially on the experimentally induced subacute hemorrhagic anemia (SHA).

Therefore, in this study, we examined the ameliorating effects of NYDBT on experimental subacute hemorrhagic anemia in rats and compared with commercial iron supplements, Ferromax™ (7.14% ferric hydroxide polymaltose complex; FM, Hanmi Pharm. Co. Ltd., Seoul, Korea).

Ⅱ. Materials and methods

1. Animals and husbandry

Total sixty healthy female SPF/VAF Crl : CD1 [Sprague-Dawley] rats (6 weeks old upon receipt; Orient Bio, Seungnam, Korea; Body weight ranged in 120~150 g upon receipt), were used after acclimatization for 8 days. Animals were assigned four to five per polycarbonate cage in a temperature (20~25℃) and humidity (50~55%) controlled room. Light : dark cycle was 12 hr : 12 hr, and standard rodent chow (Samyang, Seoul, Korea) and water were supplied free to access. After 8 days after acclimatization, eight rats in each group were selected based on the body weight deviations (Average

201.52±8.13 g, ranged in 183～217 g) as follows. All laboratory animals were treated as the national regulations of the welfare and usage of laboratory animals, admitted by the Institutional Animal Care and Use Committee in Daegu Haany University (Gyeongsan, Gyeongbuk, Korea) before animal experiment (Approval No DHU2015-029, April 13, 2015).

Experimental groups (Six groups, 8 rats in each group were used)

1. Intact vehicle control : Distilled water administered intact rats
2. SHA control : SHA-induced and distilled water administered rats
3. FM : SHA-induced and FM 5 ml/kg administered rats
4. NYDBT 500 : SHA-induced and NYDBT 500 mg/kg administered rats
5. NYDBT 250 : SHA-induced and NYDBT 250 mg/kg administered rats
6. NYDBT 125 : SHA-induced and NYDBT 125 mg/kg administered rats

2. Preparations and administrations of test materials

Aqueous extracts of NYDBT (Table 1) (yield=15.17%) as brown powders were prepared by routine methods using rotary vacuum evaporator (N-1110, Eyela, Tokyo, Japan) and programmable freeze dryer (FDB-5503, Operon, Kimpo, Korea), which were purchase from local voucher (Jecheon Hanbang Yakcho, Jecheon, Korea) after confirm the morphology under microscopy. Total 494 g of NYDBT were boiled in 5 L of distilled water for 4 hrs, three times at 60℃ and evaporated using automated round flasked evaporator (Eyela N-1110, Tokyo, Japan), then lyophilized completely. Total 74.94 g (yield=15.17%) of lyophilized aqueous NYDBT extracts were acquired. NYDBT extracts were stored at -20℃ in a refrigerator to protect from light and humidity until used. The voucher specimens documenting this purchase and some specimens of lyophilized aqueous extracts of NYDBT were deposited in the herbarium of the Medical Research center for Globalization of Herbal Formulation, Daegu Haany University (Code NYDBT2015KDC). Deep brown solutions of FM (Hanmi Pharm. Co. Ltd., Seoul, Korea) were also stored at 4℃ in a refrigerator to safeguard from light and humidity until used. FM was well diluted as 1:4 (v/v) indistilled water, and NYDBT extracts were well dissolved upto 100 mg/ml in distilled water, at least in a condition of this experiment. Test articles were orally administered 5 ml/kg, once a day for 7 days at 1 hr after exsanguination. NYDBT extracts were dissolved in distilled water as 100, 50 and 25 mg/ml concentration, and orally administered in a volume of 5 ml/kg, equivalence to 500, 250 and 125 mg/kg, respectively. FM solution was diluted by distilled water as 1:4 (v/v), and orally administrated in a volume of 5 ml/kg, once a day for 7 days at 1 hr after exsanguination. In intact and SHA control rats, distilled water 5 ml/kg was orally administered, instead of test substance.

Table 1. Composition of NYDBT

Herbs	Scientific name	Korean name	Amounts (g)
Cistanchis Herba	Cistanche deserticola Y.C.Ma	肉蓯蓉	4
Eucommiae Cortex	Eucommia ulmoides Oliver	杜　仲	4
Paeoniae Radix Alba	Paeonia lactiflora Pallas	白芍藥	2.8
Atractylodis Rhizoma Alba	Atractylodes macrocephala Koidzumi	白　朮	2.8
Aconiti Lateralis Radix Preparata	Aconitum carmichaeli Debe	附　子	2.8
Ginseng Radix Alba	Panax ginseng C.A.Meyer	人　參	2.8
Cinnamomi Cortex Spissus	Cinnamomum cassia Presl	肉　桂	2.8
Pinelliae Tuber	Pinellia ternata Breitenbach	半　夏	2.8
Dendrobii Herba	Dendrobium nobile Lindley	石　斛	2.8
Schisandrae Fructus	Schisandra chinensis Baillon	五味子	2.8
Cervi Parvum Cornu	Cervus nippon Temminck	鹿　茸	2
Astragali Radix	Astragalus membranaceus Bunge	黃　耆	2
Angelicae Gigantis Radix	Angelica gigas Nakai	當　歸	2
Poria (Hoelen)	Poria cocos (Schw.) Wolf	白茯苓	2
Rehmanniae Radix Preparata	Rehmannia glutinosa (Gaert-ner) Liboschitz ex Steudel	熟地黃	2
Glycyrrhizae Radix	Glycyrrhiza uralensis Fischer	甘　草	1
Zingiberis Rhizoma Crudus	Zingiber officinale Roscoe	生　薑	4
Zizyphi Fructus	Zizyphus jujuba Miller var.*i-nermis* Rehder	大　棗	4
Total	18 types		49.4

3. Induction of SHA

SHA in rats was induced according to the previous studies[14] with some modifications. Briefly, animals were anesthetized with 2 to 3% isoflurane (Hana Pharm. Co., Hwasung, Korea) in the mixture of 70% N_2O and 28.5% O_2, exsanguinations of whole bloods 1 ml/head, match to about 0.5% of body weights, were continuously executed from orbital plexus, once a day for 7 days in each rats, at 1 hr before test material administration. In intact vehicle control rats, no exsanguinations were conducted but system inhalation anesthesia was also conducted in this experiment.

4. Changes in body weights

Changes of body weight were checked at once a day from 1 day before initial exsanguinations and test material throughout all periods of experiment using an automatic electronic balance (Precisa Instrument, Zuerich, Switzland). To reduce the differences of individuals, the body weight gains after 7 days of administrations were calculated as follow Equation [1].

Equation [1]. Body Weight Gains (g) during 7 days of test article treatment from initiation of test article administrations to end of 7 days of test article administrations = Body weights at sacrifice (24 hrs after last administrations and exsanguinations) -body weights at start of administrations

or exsanguinations

5. Organ weight measurements

At sacrifice, the weights of spleen, liver and left femur were measured at g levels as absolute wet-weights and to reduce the differences of individual body weights, the relative weights (% of body weights) were also computed using body weight at sacrifice and absolute weight as follow Equation [2].

Equation [2]. Relative Organ Weights (% of body weight)

=(Absolute spleen, liver or femur weights /Body weight at sacrifice)×100

6. Hematology

Blood samples were drawn from posterior vena cava using a syringe with a 23 gauge needle under 2 to 3% isoflurane inhalation anesthesia in the mixture of 70% N_2O and 28.5% O_2. The animals had been 18 hrs overnight fasted (water was not restricted) prior to sacrifice and blood collecting. The blood samples were collected into CBC bottles containing EDTA-2K (1.8 mg/ml of blood). Items for hematology measurement are listed below and the detecting methods and unit measured are presented as follow items, respectively. All hematological measurements were conducted in Veterinary Teaching Hospital, College of Veterinary Medicine, Kyungpook National University (Daegu, Korea) using automated hematology cell counter (MS9-5V; Melet Schloesing Lab., Paris, France).

7. Measurement of intestinal charcoal transit ratio

Assessment of gastrointestinal propulsion of charcoal meal was determined as Sagar *et al*[15] with minor modifications[16]. Test animals were starved for 18 hrs before the experiment, but consumed water *ad libitum*. Ten minutes after last 7th test material administration, animals from each group were fed on 1 ml of charcoal meal (3% suspension of activated charcoal in 0.5% aqueous methylcellulose (Sigma -Aldrich, St. Louise, MO, USA)). After thirty minutes of administrating of charcoal meals, the animals of each groups were killed by cervical dislocation. And then, total small intestine length (pyloric sphincter to caecum) and the distance which the charcoal moved as a fraction of that length was measured. The intestinal charcoal transit ratio was calculated as the difference between the total small intestinal length and length of charcoal meal transferred as Equation [3][17].

Equation [3]. Charcoal Transit Ratio (%)

=(Length of charcoal meal transferred /Total small intestine length)×100

8. Smear cytology

At sacrifice, 0.5 ml of whole bloods were collected from orbital plexus under inhalation anesthesia, and directly smeared on slide. Preparations were dried, and fixed by submerging in absolute methanol (for 30 minutes). Fixed slides were stained by 1:6 diluted Giemsa solutions (Sigma -Aldrich, St. Louise, MO, USA) for 10

minutes. Slides were randomly coded and examined under 400 times magnification by two different experts. Mean RBC diameters (μm/cell) and mean PCEs (PCE/1000 cells) were observed using a computer-assisted image analysis program (iSolution FL ver 9.1, IMT i-solution Inc., Vancouver, Quebec, Canada) to decide whether microcytic or macrocytic anemia, regenerative or degenerative anemia[1,18].

9. Histopathology

The left femur, left lateral lobes of liver and spleen were sampled at 7 days after first exsanguinations and after measurement of charcoal transfer, and fixed in 10% neutral buffered formalin (NBF). After fixation, femur samples were decalcified using decalcifying solution [24.4% formic acid, and 0.5N sodium hydroxide] for 5 days (mixed decalcifying solution was exchanges once a day for 5 days), not in liver and spleen samples. After that, they were lengthways trimmed and embedded in paraffin, sectioned (3～4 μm) and stained with Hematoxylin & Eosin (H&E). The histological profiles of the femur bone marrow regions, hepatic parenchyma has focused on the hematopoietic spots, spleen red pulps were observed as compared with the SHA and intact vehicle control. The mean numbers of total femur bone marrow cells ($\times 10^3$ cells/mm^2), area of hematopoietic spots in the liver parenchyma (%/mm^2) and numbers of total spleen red pulp cells ($\times 10^3$ cells/mm^2) were measured as histomorphometrical analyses at prepared longitudinally trimmed samples using a computer-assisted image analysis program. In addition, assessment of histological observations of colon mucosa and fecal pellets remnant in the colon lumen were determined according to Wu et al[19] with minor modifications[16]. Briefly, the segments of rat distal colon containing one fecal pellet were seperated by ligatures, removed, and immediately fixed with 10% NBF at intestinal charcoal transit ratio measurement. The fixed tissue segments were embedded in paraffin and serially cut into 3 μm thick cross sections. The sections were stained with alcian blue at pH of 2.5. Eight tissue segments per group were prepared and, the histological profiles were interpreted as mean thickness of mucosal layers at the fecal surface (μm/fecal pellets), mucous-producing goblet cell (alcian blue positive cell) numbers (cells/mm^2 of colonic mucosa) and colonic mucosa thicknesses (μm/colon) using a computer-assisted image analysis program. The histopathologist was blinds to group distribution when this analysis was made.

10. Immunohistochemistry

The changes of CD34 and CD45-immunoreactive cells, the hematopoietic stem cells, were observed by immunohistochemistrical methods using purified rat anti-mouse CD34 and CD45 antibodies (BD Biosciences, San Jose, CA, USA) with avidin-biotin-peroxidase (ABC) and peroxidase substrate kit (Vector Labs, Burlingame, CA, USA). Briefly, endogenous

peroxidase activity was cut off by incubated in methanol and 0.3% H_2O_2 for 30 minutes. Non-specific binding of immunoglobulin was cut off with normal horse serum blocking solution for 1 hr in humidity chamber after heating (95~100℃) based epitope retrievals in 10 mM citrate buffers (pH 6.0) in spleen and liver samples or by pretreatment of trypsin (Sigma-Aldrich, St. Louise, MO, USA) and 2NHCl in femur samples[20]. Primary antisera was treated for overnight at 4℃ in humidity chamber, and then incubated with biotinylated universal secondary antibody and ABC reagents for 1 hr at room temperature in humidity chamber. Finally, reacted with peroxidase substrate kit for 3 minutes at room temperature. All sections were rinse in 0.01 M PBS for 3 times, between each step. The cells taken by over 20% of immunoreactivities, the density of CD34 and CD45, were regarded as positive. The mean numbers of CD34 and CD45-immunoreactive cells dispersed in the liver, spleen and femur bone marrow (mm^2) were counted using a computer -assisted image analysis program, respectively. The histopathologist was blinded to the group distribution when performing the analysis.

11. Statistical analyses

All Data was expressed as mean± standard deviations (SD) of eight rats. Multiple comparison tests for all dose groups were treated. Variance homogeneity was examined using the Levene test[21]. If the Levene test signified no significant deviations from variance homogeneity, the obtain data was analyzed by one way ANOVA test followed by least-significant differences multi-comparison (LSD) test to determine which pairs of group comparison were significantly different. In case of significant deviations from variance homogeneity were observed at Levene test, a non-parametric comparison test, Kruskal-Wallis H test was conducted. When a significant difference is observed in the Kruskal-Wallis H test, the Mann -Whitney U (MW) test was conducted to determine the specific pairs of group comparison, which are significantly different. Statistical analyses were conducted using SPSS for Windows (Release 14.0K, IBM SPSS Inc., Armonk, NY, USA). In addition, the percent changes between intact vehicle and SHA control rats were calculated to observe the severities of SHA induced by continuous exsanguinations in this study, and the percent changes as compared with SHA control and test material treated rats were also calculated to help the understanding of the anti-anemic effects of test substances as follow Equation [4] and [5], according to previous method described by Kang *et al*[22], respectively.

Equation [4]. Percent Changes as Compared with Intact Vehicle Control (%)

={(Data of SHA control-Data of intact vehicle control rats)/Data of intact vehicle control rats}×100

Equation [5]. Percent Changes as Compared with SHA Control (%)

={(Data of test material treated rats -Data of SHA control)/Data of SHA control}×100

Ⅲ. Results

1. Effects on body weights

Significant ($p<0.01$) decreases of body weights were detected in SHA control from 3 days after exsanguinations as compared with intact vehicle control, consequently, the body weight gains during 7 days of treatment period were also significantly ($p<0.01$) decreased. No meaningful or significant changes on the body weight and gains were observed in FM treated rats as compared with SHA control rats, throughout the whole experimental periods, but NYDBT 500, 250 and 125 mg/kg treated rats showed significant ($p<0.01$ or $p<0.05$) increased body weights from 4, 5 and 6 days after administration, and accordingly, the body weight gains during 7 days of administration periods were also significantly ($p<0.01$) increased in all three different dosages of NYDBT administered rats as compared with SHA control rats, respectively (Table 2).

Table 2. Changes on the Body Weights

Groups \ Items	Body weights (g)			Body weight gains [B-A]
	Before treatment	At initial treatment [A]*	At last treatment [B]*	
Controls				
Intact	201.25±6.94	181.13±8.95	211.25±4.98	30.13±6.79
SHA	201.63±10.13	180.25±10.14	186.38±7.33[a]	6.13±6.69[a]
FM 5 ml/kg	201.50±10.30	179.38±10.39	187.00±8.91[a]	7.63±3.07[a]
NYDBT				
500 mg/kg	201.38±7.23	180.63±9.30	208.00±6.00[c]	27.38±6.95[c]
250 mg/kg	201.50±8.55	181.50±10.41	202.88±9.99[bc]	21.38±3.54[ac]
125 mg/kg	201.88±7.86	181.88±9.78	198.88±3.91[ac]	17.00±6.39[ac]

Values are expressed mean±SD of eight rats.
* : All animals were overnight fasted.
a : $p<0.01$ and b : $p<0.05$ as compared with intact vehicle control by LSD test
c : $p<0.01$ as compared with SHA control by LSD test

2. Changes on the spleen weights

Significant ($p<0.01$) increases of absolute and relative spleen weights were demonstrated in SHA control rats as compared with intact vehicle control rats. However, significant ($p<0.01$) decreases of spleen weights were noticed in NYDBT 500, 250 and 125 mg/kg treated rats as compared with SHA control rats, but not in FM treated rats, in this experiment (Table 3).

3. Changes on the liver weights

Significant ($p<0.01$ or $p<0.05$) decreases of absolute and relative liver weights were demonstrated in SHA control rats as compared with intact vehicle control, respectively. FM

treatment did not influenced on the hepatic weights as compared with SHA control rats. Significant ($p<0.01$ or $p<0.05$) increases of absolute liver weights were demonstrated in all three different dosages of NYDBT 500, 250 and 125 mg/kg treated rats as compared to those of SHA control rats, but not in relative liver weights (Table 3).

4. Changes on the femur weights

Significant ($p<0.01$) decreases of absolute femur weights were demonstrated in SHA control rats as compared with intact control. Anyway, no meaningful changes on the femur weights were demonstrated in all test substance treated rats as compared with SHA control rats, except for significant ($p<0.01$) increases of absolute femur weights detected in NYDBT 500 mg/kg treated rats as compared with SHA control rats, respectively (Table 3).

Table 3. Changes on the Spleen, Liver and Femur Weights

Groups \ Items	Absolute wet-weights (g)			Relative wet-weights (% of body weight)		
	Spleen	Liver	Femur	Spleen	Liver	Femur
Controls						
Intact	0.555±0.038	6.658±0.261	0.660±0.035	0.263±0.022	3.152±0.108	0.312±0.017
SHA	0.680±0.024[a]	5.455±0.337[e]	0.606±0.043[a]	0.365±0.020[a]	2.930±0.199[b]	0.326±0.031
FM 5 ml/kg	0.669±0.038[a]	5.727±0.364[e]	0.612±0.017[a]	0.359±0.030[a]	3.066±0.203	0.328±0.021
NYDBT						
500 mg/kg	0.605±0.042[ac]	6.238±0.210[ef]	0.657±0.030[c]	0.291±0.022[bc]	3.002±0.162	0.316±0.016
250 mg/kg	0.631±0.020[ac]	6.047±0.124[ef]	0.632±0.036	0.312±0.024[ac]	2.989±0.197	0.312±0.021
125 mg/kg	0.645±0.012[ad]	5.879±0.166[eg]	0.618±0.035[b]	0.324±0.006[ac]	2.958±0.118[b]	0.311±0.019

Values are expressed mean±SD of eight rats.
a : $p<0.01$ and b : $p<0.05$ as compared with intact vehicle control by LSD test
c : $p<0.01$ and d : $p<0.05$ as compared with SHA control by LSD test
e : $p<0.01$ as compared with intact vehicle control by MW test
f : $p<0.01$ and g : $p<0.05$ as compared with SHA control by MW test

5. Changes on the hematology

Significant ($p<0.01$) decreases of RBC numbers, Hct and Hb and increases of PLT numbers, μRBC and MRBC ratios were observed in SHA control rats as compared with intact vehicle control rats, respectively. However, these abnormal changes on the hematological inspections were significantly ($p<0.01$) normalized by treatment of all test substances except for PLT numbers. No meaningful changes on the WBC numbers, MCV, MCH and MCHC were noticed in all SHA-induced rats, and also no significant changes on the MRBC ratios were noticed in FM 5 ml/kg, NYDBT 500, 250 and 125 mg/kg administered rats as compared with SHA control rats, in the present study (Table 4).

Table 4. Changes on the Hematological Values

Groups / Items	Controls		FM 5 ml/kg	NYDBT		
	Intact	SHA		500 mg/kg	250 mg/kg	125 mg/kg
WBC (K/μl)	14.86±2.64	14.81±2.40	14.44±2.49	14.83±1.24	15.04±2.61	14.70±1.63
RBC (M/μl)	6.76±0.45	4.23±0.45[a]	5.10±0.26[ac]	6.14±0.34[ac]	5.66±0.45[ac]	5.28±0.27[ac]
MCV (fl)	54.26±1.92	54.08±2.10	54.49±1.29	54.33±2.01	54.38±2.39	54.01±2.79
HCT (%)	36.59±3.61	23.46±1.19[a]	28.39±2.29[ac]	34.08±1.00[bc]	31.89±1.26[ac]	29.40±2.92[ac]
MCH (pg)	28.18±1.56	29.18±1.12	28.53±1.32	28.44±1.96	28.34±2.09	28.85±1.91
MCHC (g/dl)	52.61±1.43	53.41±1.56	53.00±1.53	53.20±1.38	53.19±1.00	53.35±1.38
Hb (g/dl)	18.96±0.68	13.86±0.81[a]	15.79±0.71[ac]	18.25±0.63[c]	17.24±1.00[ac]	16.70±0.84[ac]
μRBC (%)	1.69±0.29	3.18±0.22[a]	2.53±0.27[ac]	1.99±0.19[bc]	2.14±0.18[ac]	2.34±0.24[ac]
MRBC (%)	1.53±0.18	2.11±0.48[a]	2.06±0.24[a]	1.94±0.32[b]	2.01±0.36[a]	1.94±0.18[b]
PLT (M/μl)	754.38±82.86	994.50±80.31[a]	971.88±90.02[a]	948.00±109.75[a]	981.25±65.87[a]	974.25±65.33[a]

Values are expressed mean±SD of eight rats.
a : $p<0.01$ and b : $p<0.05$ as compared with intact vehicle control by LSD test
c : $p<0.01$ as compared with SHA control by LSD test

6. Changes on the gastrointestinal motility

Although no significant changes on the charcoal transfer rates, the digestive motility, were detected in SHA control rats and all three different dosages of NYDBT administered rats as compared with intact vehicle control rats, significant ($p<0.01$) decreases of charcoal transfer rates in gastrointestinal tract were observed in FM 5 ml/kg treated rats as compared with intact and SHA vehicle control rats, in this study (Table 5).

Table 5. Changes on the Gastrointestinal Motility

Items / Groups	Gastrointestinal motility		
	Total small intestine length (cm) [A]	Length of charcoal meal transferred (cm) [B]	Charcoal transfer rates (%) [B/A×100]
Controls			
Intact	108.25±8.14	81.88±5.08	75.94±6.47
SHA	107.38±7.82	79.75±8.40	74.63±9.40
FM 5 ml/kg	105.88±9.11	52.75±10.39[ab]	49.89±9.32[ab]
NYDBT			
500 mg/kg	107.25±4.74	80.75±9.10	75.37±8.74
250 mg/kg	110.50±5.48	83.38±6.99	75.46±5.45
125 mg/kg	108.13±2.80	82.88±7.47	76.60±5.90

Values are expressed mean±SD of eight rats.
a : $p<0.01$ as compared with intact vehicle control by LSD test
b : $p<0.01$ as compared with SHA control by LSD test

7. Changes on the blood smear cytology

Significant ($p<0.01$) decreases of mean RBC diameters and increases of PCEs were observed in SHA control rats as compared with intact vehicle control rats, respectively. However, these abnormal changes on the smear cytological inspections were significantly ($p<0.01$) normalized by treatment of all test substances including FM 5 ml/kg, in this result.

8. Changes on the femur bone marrow histopathology

Significant ($p<0.01$) increases of total femur bone marrow cell numbers and decreases of CD34 and CD45 immunolabeled cells were observed in SHA control rats as compared with intact vehicle control rats, respectively. Significant ($p<0.01$) decreases of total femur bone marrow cell numbers and marked increases of CD34 and CD45 immunoreactive cells were observed in all test substance treated rats as compared with SHA control rats, in the current study (Table 6).

Table 6. Changes on the Femur Bone Marrow Histomorphometrical Analysis

Groups \ Items	Femur bone marrow histomorphometry		
	Mean numbers of total cells ($\times10^3$ cells/mm^2)	Mean numbers of CD34+ cells (cells/mm^2)	Mean numbers of CD45+ cells (cells/mm^2)
Controls			
Intact	16.76±1.10	108.88±19.87	127.63±22.09
SHA	55.21±10.25[c]	14.38±2.97[a]	33.75±8.17[a]
FM 5 ml/kg	43.28±3.24[cd]	40.88±11.47[ab]	41.88±5.54[a]
NYDBT			
500 mg/kg	32.06±4.75[cd]	89.50±17.35[ab]	91.88±21.05[ab]
250 mg/kg	38.58±5.52[cd]	75.50±11.19[ab]	83.25±10.28[ab]
125 mg/kg	41.58±5.70[cd]	67.50±11.48[ab]	73.63±11.41[ab]

Values are expressed mean±SD of eight rats.
a : $p<0.01$ as compared with intact vehicle control by LSD test
b : $p<0.01$ as compared with SHA control by LSD test
c : $p<0.01$ as compared with intact vehicle control by MW test
d : $p<0.01$ as compared with SHA control by MW test

9. Changes on the liver histopathology

Significant ($p<0.01$) increases of the area occupied by hematopoietic spots and decreases of CD34 and CD45 immunolabeled cells were observed in SHA control rats as compared with intact vehicle control rats, respectively. Significant ($p<0.01$ or $p<0.05$) decreases of the area percentages occupied by hematopoietic spots and increases of portal triad CD34 and CD45 immunoreactive cells were observed in all test substance treated rats as compared with SHA control rats, in the current experiment (Table 7).

Table 7. Changes on the Liver Histomorphometrical Analysis

Groups \ Items	Liver histomorphometry: Mean area of hematopoietic spots (%/mm^2)	Mean numbers of CD34+ cells (cells/mm^2)	Mean numbers of CD45+ cells (cells/mm^2)
Controls			
Intact	1.63±0.90	173.75±16.65	260.75±44.68
SHA	29.00±5.81[d]	68.00±12.28[d]	104.38±20.30[a]
FM 5 ml/kg	16.98±2.35[df]	93.63±11.25[df]	145.88±10.38[ac]
NYDBT			
500 mg/kg	6.93±2.82[df]	253.63±53.13[df]	280.25±45.56[b]
250 mg/kg	12.58±3.14[df]	215.88±42.96[ef]	234.38±41.69[b]
125 mg/kg	15.41±1.91[df]	165.25±22.50[f]	193.88±51.20[ab]

Values are expressed mean±SD of eight rats.
a : $p<0.01$ as compared with intact vehicle control by LSD test
b : $p<0.01$ and c: $p<0.05$ as compared with SHA control by LSD test
d : $p<0.01$ and e: $p<0.05$ as compared with intact vehicle control by MW test
f : $p<0.01$ as compared with SHA control by MW test

10. Changes on the spleen histopathology

Significant ($p<0.01$) increases of the total red pulp cell numbers and decreases of CD34 and CD45 immunolabeled cells were observed in the spleen of SHA control rats as compared with intact vehicle control rats, respectively. However, significant ($p<0.01$ or $p<0.05$) decreases of the total red pulp cells and increases of red pulp CD34 and CD45 immunoreactive cells were observed in all test substance treated rats including NYDBT 125 mg/kg administered rats as compared with SHA control rats, in this study (Table 8).

Table 8. Changes on the Spleen Histomorphometrical Analysis

Groups \ Items	Spleen histomorphometry: Total red pulp total cells (×10^{3}cells/mm^2)	Mean numbers of CD34+ cells (cells/mm^2)	Mean numbers of CD45+ cells (cells/mm^2)
Controls			
Intact	2.77±0.71	163.50±21.57	141.13±29.58
SHA	21.22±2.68[a]	31.25±10.40[e]	29.50±12.02[a]
FM 5 ml/kg	14.66±1.81[ac]	63.25±12.74[eg]	70.63±23.60[ad]
NYDBT			
500 mg/kg	10.40±1.90[ac]	278.50±70.06[eg]	252.50±32.96[ac]
250 mg/kg	12.30±1.77[ac]	203.50±30.72[fg]	106.00±41.68[bc]
125 mg/kg	14.22±1.53[ac]	192.63±32.23[fg]	96.25±33.61[ac]

Values are expressed mean±SD of eight rats.
a : $p<0.01$ and b : $p<0.05$ as compared with intact vehicle control by LSD test
c : $p<0.01$ and d : $p<0.05$ as compared with SHA control by LSD test
e : $p<0.01$ and f : $p<0.05$ as compared with intact vehicle control by MW test
g : $p<0.01$ as compared with SHA control by MW test

11. Changes on the histopathology of colon and remnant feces

No meaningful or significant changes on the histopathological profiles of the colon and remnant feces *in situ*, the surface mucosa thicknesses of remnant fecal pellets in the colon lumen, mucosa thicknesses and the mucous producing cell numbers in the colonic mucosa were demonstrated in SHA control and all three different dosages of NYDBT treated rats as compared with intact vehicle control, respectively. However, significant ($p<0.01$) decreases of the surface mucous thicknesses of remnant fecal pellets in the colon lumen, the mucosa thicknesses and mucous producing cell numbers in the colon were detected in FM 5 ml/kg treated rats as compared with intact vehicle and SHA control rats, in the present study (Table 9).

Table 9. Changes on the Histomorphometrical Analysis of the Colon and Remnant Fecal Pellets *in situ*

Groups \ Items	Colon and remnant fecal histomorphometry		
	Fecal surface mucous thickness (μm)	Number of mucosal goblet cells (cells/mm^2)	Mean thickness of colonic mucosa (cells/mm^2)
Controls			
Intact	30.91±12.33	435.25±128.58	190.69±40.23
SHA	31.38±12.05	447.50±108.77	189.96±38.82
FM 5 ml/kg	13.54±4.70^{ab}	205.25±62.51^{ab}	125.62±19.99^{ab}
NYDBT			
500 mg/kg	34.22±10.45	470.38±152.34	190.19±44.01
250 mg/kg	31.24±7.33	480.13±106.30	196.69±42.68
125 mg/kg	33.57±6.89	482.75±92.94	191.94±21.72

Values are expressed mean±SD of eight rats.
a : $p<0.01$ as compared with intact vehicle control by LSD test
b : $p<0.01$ as compared with SHA control by LSD test

Ⅳ. Discussion

Iron deficiency anemia is a common anemia because of chronic or subacute blood losses including in case of women with menorrhagia or menometrorrhagia. It has been treated by iron supplements, but can induce digestive discomfort, especially constipation. Therefore, new treatments have to be concerned on natural herbs less toxic effects than iron supplements.

We can consider a traditional medicine prescription used in blood deficiency[7]. NYDBT is a traditional medicine that has been used for several hundred years, predominantly to tonic purpose in a weak constitution as qi and blood deficiency or ameliorating loss of blood of women[8].

Considerable amounts of bleeding leads hypovolemic situations in mammals, and accordingly marked decreases of body weights were accompanied[23]. In the present

study, noticeable decreases of body weights were also induced by continuous exsanguinations of 1 ml/head (about 0.5% body weights), once a day, from forth bleedings in SHA control rats, with significant decreases of body weight gains after seven repeated exsanguinations as secondary results from hypovolemic situations considering the facts that indicated by other investigators[23]. In the present study, NYDBT dose-dependently and significantly inhibited the hypovolemic related decreases of body weights, but not in FM 5 ml/kg treatment. These results are suggested that NYDBT can be ameliorating the body weight losses induced by SHA through their hematopoietic activities.

Iron deficient and regenerative anemia have been showed marked decreases of RBC, Hct and Hb with increases of μ RBC and MRBC and peculiarly noticeable increases of PLT were also accompanied in subacute or chronic iron deficient and regenerative anemia on the hematological observations[24]. In the present study, rats exsanguinations of 1 ml/head, once a day for 7 days, also showed classic SHA related hematological changes including decreases of RBC, Hct and Hb with increases of PLT, μRBC and MRBC, respectively. These SHA related hematological changes were effectively inhibited by oral treatment of commercial iron supplements, FM and also by oral administrations of all three different dosages of NYDBT, dose-dependently, except for PLT, MRBC numbers.

On the smear cytological aspects, classic microcytic and hypochromatic anemia were observed in hemorrhage related regenerative anemia[1,18], and also decreases of RBC diameters and increases of PCEs were detected in SHA control rats of the present study, as typical regenerative and iron deficient anemia. These smear cytological changes were also favorably inhibited by oral treatment of FM 5 ml/kg, and also dose-dependently in NYDBT 500, 250 and 125 mg/kg administered rats, respectively.

Continuous bleeding related hemorrhagic anemia induced excessive erythropoiesis in the bone marrow with start of extramedullary ectopic hematopoiesis in fetal hematopoietic organs, liver and spleen[25]. Marked and significant increases of total femur bone marrow cells, increases of hematopoietic spots in liver and increases of splenic red pulp cells, may be indicated ectopic hematopoiesis were also observed in SHA control rats, in the present study, with noticeable increases of spleen weights. Once again, these ectopic hematopoiesis and bone marrow hyperplasia were meaningfully inhibited by treatment of all test substances used in this study.

In chronic anemia, depletion of hematopoietic stem cells, the decreases of absolute cell numbers, have been observed related to over differentiation into erythrocyte precursor cells or migration into peripheral bloods with premature erythrocytes like MRBC or PCEs[26]. In the present study, CD34+ and CD45+

cells were regarded as hematopoietic stem cells on the basis of the previous reports[24], marked decreases of hematopoietic stem cells were detected in the femur bone marrow, splenic red pulp and hepatic portal triad regions after immunohistochemistrical analysis using CD34 and CD45 antibodies, may be related to the over differentiations or migration considering the report of other investigators[26]. However, these SHA-related decreases of hematopoietic stem cells were marked and favorably inhibited by treatment of FM and all three different dosages of NYDBT. Obvious dose-dependently inhibitions of the CD34+ and CD45+ cells were observed in NYDBT treated rats in all three observed hematopoietic organs - the femur bone marrow, spleen and liver. However, the possibilities that NYDBT also potentially inhibited the migration of stem cells, were also could not completely exclude in the present study. Therefore, more fundamental mechanism studies should be tested in future.

The transit process of the entire gastrointestinal tract reflected the overall gastrointestinal motor activity, and measuring gastrointestinal charcoal transit ratio is useful in diagnosis of constipation[27]. Since the decrease of gastrointestinal charcoal transit ratio means constipation[15,16], the decreased gastrointestinal charcoal transits induced by treatment of FM 5 ml/kg, detected in this study, were considered as one of direct evidences that commercial iron supplement used in this experiment induced the digestive discomfort, constipation, well corresponded to the previous reports[4]. In addition, reduces of mucous production on the colonic mucosa are directly related with constipation[15,16], and marked decreases of colonic mucosa layer thicknesses and mucous producing cells has been detected in animals suffering from constipation at histopathology[16]. Moreover, FM 5 ml/kg also induced noticeable and significant decreases of mucous components in the colon and remnant feces *in situ* under alcian blue staining as direct evidences that they induced constipation. However, no meaningful changes on the charcoal transfers, mucous components in the colonic mucosa and also in remnant feces were demonstrated in all three different dosages of NYDBT as compared to those of intact vehicle and SHA control in this experiment. These are direct evidences that NYDBT has potent favorable anti-anemic effects on SHA through proliferating effects on hematopoietic stem cells with less digestive discomfort, constipations, one of major problems in iron supplements[4].

The results obtained in this study suggest that oral administration of NYDBT 500, 250 and 125 mg/kg has clear dose-dependently favorable anti-anemic in SHA rats through proliferating effects on hematopoietic stem cells with less digestive discomfort at least, in a condition of this experiment. It, therefore, is expected that NYDBT will be promising as a novel alternative hematopoietic and therapeutic agent for anemia. Since NYDBT consisted of 18 herbs and each herb has various active

ingredients, the screening of the biological active compounds should be conducted in future with more detail mechanism studies.

Ⅴ. Conclusion

In this study, we reached the following results:

1. NYDBT dose-dependently and significantly inhibited the hypovolemic related decreases of body weights, but not in FM 5 ml/kg treatment.
2. Significant decreases of spleen weights, increases of absolute liver weights were noticed in NYDBT treated rats, but not in FM treated rats. In case of femur weights, significant increases of absolute femur weights were only demonstrated in NYDBT 500 mg/kg.
3. More favorable and significant increases of RBC numbers, Hct and Hb and decreases of μRBC ratios were demonstrated in FM 5 ml/kg and NYDBT dose-dependently.
   And also, more favorable and significant increases of RBC diameters and decreases of PCEs were demonstrated in FM 5 ml/kg and NYDBT dose-dependently.
4. Obvious increases of the CD34+ and CD45+ cells were observed in FM 5 ml/kg and NYDBT dose-dependently treated rats in all three observed hematopoietic organs - the femur bone marrow, spleen and liver.
5. Significant decreases of gastrointestinal charcoal transits, mucous components in the colon and remnant feces *in situ* were induced by treatment of FM 5 ml/kg only. However, no meaningful changes on all three different dosages of NYDBT.

According to these results, oral administrations of NYDBT 500, 250 and 125 mg/kg has clear dose-dependently anti-anemic effect on SHA rats.

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국문초록

목 적: 본 연구는 녹용대보탕이 안와정맥총 방혈로 유발시킨 랫트의 아급성 출혈성 빈혈 모델에 대해 항빈혈 및 조혈촉진 효과를 확인하였다.

방 법: 본 실험에서는 안와 정맥총에서 혈액을 채혈하여 아급성 출혈성 빈혈을 유발시켰으며, 녹용대보탕 추출물을 경구투여하고, 체중 및 조혈 관련 장기(대퇴골 골수, 비장 및 간) 중량, 적혈구 관련 혈액학적 지표의 변화, 혈액 도말 표본의 세포학적 변화, 조혈관련 장기의 조직병리학적 변화와 함께 조혈장기 내 혈구형성 줄기세포의 수적 변화를 면역조직화학적으로 관찰하였다. 또한 철분 보충제의 대표적 부작용인 소화 장애, 특히 변비 유발 여부를 확인하기 위해 탄분을 이용해 위장관 운동성을 평가하였으며, 결장내 분변 표면의 점막 두께와 결장점막 내 점액생산세포 및 결장 점막 두께 변화를 조직병리학적으로 평가하였다.

결 과: 본 실험에서 유발된 아급성 출혈성 빈혈 소견이 모든 세 용량의 녹용대보탕 추출물의 용량 의존적으로 현저히 억제되었다. 또한 세 용량의 녹용대보탕 투여군에서는 정상 대조군 및 아급성 출혈성 빈혈 대조군에 비해 위장관 운동성 변화 및 결장 및 결장내 분변의 점액성분의 유의미한 변화는 없었다.

결 론: 이상의 결과에서, 녹용대보탕의 경구투여는 아급성 출혈성 빈혈 유발 랫트에서 단순 철분 보충제의 부작용인 소화관 장애 없이, 조혈관련 장기들에서 혈구형성줄기세포의 증식 촉진을 통해 투여 용량 의존적으로 철 결핍성 빈혈의 증상을 현저히 완화시키는 것으로 관찰되었다. 따라서 녹용대보탕은 새로운 개념의 조혈촉진 빈혈치료제로서의 개발 가치가 충분할 것으로 판단된다.

중심단어: 녹용대보탕, 훼로맥스, 아급성 출혈성 빈혈, 조혈 효과

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