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CHOI Chang Woo, SHIN Jang Woo*, CHO Jung Hyo, SON Chang Gue, Lee Yong Yeon, LEE Yeon Weol, YOO Hwa Seung, LEE Nam Heon, Yun Dam Hee, CHO Chong Kwan

Department of Internal Medicine College of Oriental Medicine, Daejon University, Daejeon, Korea
Institute of Traditional Medicine and Bioscience, Daejeon. University, Daejeon, Korea*

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ABSTRACT

Aim: To examine the efficacy of Hominis Placenta Hydrate (HPH) on the hemopoiesis in a myelosuppression model system.

Methods: Mice were injected with 5-Fluorouracil (5-FU) intraperitoneally and were administered with 200 mg/kg and 1000 mg/kg of HPH. Peripheral blood was analyzed at 5, 9, and 13 days. Histopathologic examination of bone marrow was performed at 5 days after 5-FU injection. The expression of cytokine involved in hemopoiesis was examined by using ELISA kit.

Results: The hematology data demonstrated that the treatment of all the agents augmented monocytes and leucocytes counts in the peripheral blood WBC and platelet in HPH treated group were significant increased compared with control group. Also, cell numbers of RBC and Hb were restored. In HPH treated group, expression of IL-3, GM-CSF was significant increased But not TPO.

Conclusions: Based on the above results, it is suggested that Hominis Placenta Hydrate is an effective remedy for the bone marrow failure and myelosuppression occurring during chemotherapy

Key Words: Hominis Placenta hemopoiesis; IL-3; GM-CSF

INTRODUCTION

Stem cells provide the great hope for numerous areas, such as embryonic development,

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· 교신저자 : CHO Chong Kwan 대전 중구 대흥동
대전대학교 부속한방병원 내과
전화 : 82-42-470-9661 Fax : 82-42-229-6968.
E-mail : longrain@dju.ac.kr

differentiation, nuclear cloning, cell transplantation, tissue engineering, gene discovery, drug screening, and so on.Among various forms of stem cells, hematopoietic stem cells (HSCs) have the capacity for both self-renewal and derivation of all the blood cell lineages. Consequently, toxicity to these cells can result in neutropenia, erythrocytopenia, thrombocytopenia, pancytopenia, or aplastic anemia. Many anticancer drugs adversely affect the bone marrow (BM), and neutropenia is a common limiting factor in dose escalation. Myelosuppression is a main life-threatening side effect among them in cancer patients[1-5].

Recently, there has been enormous progress in many areas in hematology including the mechanisms of hematopoiesis, the role of cytokines, the regulation of genes and of cell growth, the functioning of the immune system, and the understanding of hemostasis and thrombosis. In accordance with discovery mechanisms of cellular transduction in BM, new medical therapeutics have been developed. For example, erythropoietin (EPO) and various colony stimulating factors have been used for the treatment of anemia associated with renal diseases and neutropenia of various causes. However, their effects were reported to be limited and unexpected side-effects occasionally occurred. Because, intracellular signaling occurs through a signaling network rather than through linear signaling pathways, it is not obvious whether it is a greater advantage to inhibit signaling at the cell membrane or farther down in the signaling network/pathways[6-12].

Considering the contemporarycircumstances, the development of new drugs and therapeutics for myelosuppression is absolutely required. Hominis Placenta Hydrate (HPH) is derived from human placenta. Human placenta belongs to extraembryonic lineage and takes much part in growth of fetus. It has been considered that human placenta is related to source of life force for many centuries in Oriental Medicine field. In fact, it has been used as a medicinal stuff for a long time in Oriental Medicine. Many researchers have reported on its effects in many areas, such as pulmonary tuberculosis, asthma, sterility, psychiatric disorders and so on. However, few studies have been come out about hematopoietic effects.Moreover, its role is not clearly defined yet[13-16]. In our study, we investigated the action mechanism of HPH in peripheral bloods and BM of 5-Fluorouracil (5-FU)administered mice as a myelosuppression model system.

Materials and Methods Materials

The *Hominis Placenta* was provided from Deajeon University Oriental Hospital. Hominis placenta was hydrated in pepcin solution at pH 2.0 for 2h. hydrated solution were centrifuged for 15min at 1500rpm. Supernatant were lyophilized. 5-FU was obtained from Choongwae Pharmaceutical Co. (Korea).

Other chemical were obtained from Sigma (ST. Louise, USA)

Experimental animals Five-week-old male ICR mice were obtained from commercial animal breeder (DaeHan Biolink, Korea) and used at 5-6 weeks of age. The mice were maintained at 22± 2℃, relative humidity at 55±10% and 12h light/dark cycles and fed with commercial pellets (Samyang Feed, Korea) and tap water *ad libitum*. Myelosuppression induction In the initial phase of

the study, ICR mice were intraperitoneally injected with 200 mg/kg 5-FU. Two days later, the mice were orally administered HPH (200 mg/kg, 1,000 mg/kg) once a day for 11 consecutive days. Positive control group was injected intraperitoneally with 100 units/kg granulocyte colony-stimulating factor (G-CSF) every two days. Cell numbers of peripheral blood and BM were measured on 5th, 9th and 13th day after 5-FU treated.

Cell counts in the peripheral blood Blood was obtained by retro-orbital venous plexus sampling with heparinized capillary tube (I.D.: 1.1~1.2㎖, Chase Scientific Glass Inc., U.S.A.).Complete blood counts were determined using blood cell counter (HEMAVET, CDC Technologies Inc., U.S.A.).

Histological analysis of BM Histological analysis of BM was performed on 5th day after 5-FU treatment in ICR mice. BM was obtained by flushing femoral bones. For the histomorphological evaluation, the femerwas dissected and fixed in 10% neutral-buffered formalin. After decalcification, fixed samples were embedded in paraplast and sections of 4 ㎛ were prepared. The sections were stained with hematoxylin and eosin for histopathological examination after decalcification.

Immunoassay of cytokines involving hematopoiesis

Cytokines involving hematopoiesis wereanalyzed on 5th day after 5-FU treatment in ICR mice. Interleukins-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), and thrombopoietin (TPO) production were measured by using Quantikine^{R} M ELISA Kit (R&D system, Inc., U.S.A.).

Statistical analysis Results were expressed as the mean±standard deviation (S.D). Statistical analysis of the data was carried out by Student's *t*-test. A difference from the respective control data at the levels of $P<0.05$ and $P<0.01$ was regarded as statistically significant.

Table 1. WBC Count in 5-FU Induced Myelosuppressed Mice

Test	Group (n=6)	0 day	5th day	9th day	13th day
WBC ($10^3/\mu l$)	Normal	12.01±1.80	11.69±1.25	11.31±0.99	12.65±2.09
	Control	12.25±1.05	4.03±0.68	3.00±0.75	7.42±1.78
	HPH (200 mg/kg)	12.61±1.05	4.61±0.74*	7.59±1.28***	12.19±2.09**
	HPH (1000 mg/kg)	12.16±1.16	4.08±1.99*	4.95±1.99*	12.17±1.99**
	G-CSF (100ul/kg)	12.30±1.51	3.77±1.05*	6.24±2.64**	9.95±0.59**

ICR mice were intraperitoneally injected with 5-FU (200 mg/kg). Two days after administered with HPH (200 mg/kg, 1000 mg/kg) and G-CSF (100ul/kg). Hematologic parameters were monitored on 0st, 5th, 9th and 13th day after 5-FU injection. Each data represents the mean±S.D. of 6 mice. Statistical significance was compared with control group by t-test. (*: not significant, **: $p<0.05$, ***: $p<0.001$)

RESULTS

1. Peripheral blood count

Blood was extracted via retro-orbital venous on 0st, 5th, 9th and 13th day after 5-FU (200 mg/kg) injection. The hematological changes in the HPH and G-CSF treatment groups showed a significant increase in cell numbers of WBC and platelet compared with control group. In RBC count, control group went on decreasing from 5th day to 13th day without interruption. On the other hand, treatment groups showed a slight restoration in the final day. However there was no significant difference between control and treatment groups in Hb except for HPH (200 mg/kg) treatment group. The results were described in table 1-4.

Table 2. RBC Count in 5-FU Induced Myelosuppressed Mice

Test	Group (n=6)	0 day	5th day	9th day	13th day
RBC ($10^6/\mu l$)	Normal	8.09±0.49	8.32±0.56	8.24±0.40	8.26±0.38
	Control	8.17±0.32	8.44±0.37	6.78±0.28	5.20±0.65
	HPH (200 mg/kg)	7.91±0.41	7.60±0.64**	6.26±0.32**	7.18±1.11*
	HPH (1000 mg/kg)	8.11±0.14	7.40±1.99***	6.27±1.99**	6.42±1.99**
	G-CSF (100ul/kg)	8.03±0.37	7.71±0.20**	6.24±0.31**	6.34±0.79**

ICR mice were intraperitoneally injected with 5-FU (200 mg/kg). Two days after administered with HPH (200 mg/kg, 1000 mg/kg) and G-CSF (100ul/kg). Hematologic parameters were monitored on 0st, 5th, 9th and 13th day after 5-FU injection. Each data represents the mean±S.D. of 6 mice. Statistical significance was compared with control group by t-test. (*: not significant, **: $p<0.05$, ***: $p<0.001$)

Table 3. Hb Count in 5-FU Induced Myelosuppressed Mice

Test	Group (n=6)	0 day	5th day	9th day	13th day
Hb (g/dl)	Normal	12.13±0.54	12.55±0.31	12.30±0.78	12.25±0.65
	Control	12.00±0.58	12.05±0.54	8.70±0.43	7.95±1.00
	HPH (200 mg/kg)	12.03±0.67	11.53±0.64*	9.03±0.26*	9.00±0.18**
	HPH (1000 mg/kg)	12.07±0.64	11.55±1.99*	8.87±1.99*	8.58±1.99*
	G-CSF (100ul/kg)	12.06±0.49	11.57±0.43*	8.77±0.75*	8.58±0.57*

ICR mice were intraperitoneally injected with 5-FU (200 mg/kg). Two days after administered with HPH (200 mg/kg, 1000 mg/kg) and G-CSF (100ul/kg). Hematologic parameters were monitored on 0st, 5th, 9th and 13th day after 5-FU injection. Each data represents the mean±S.D. of 6 mice. Statistical significance was compared with control group by t-test. (*: not significant, **: $p<0.05$, ***: $p<0.001$)

Table 4. Platelet Count in 5-FU Induced Myelosuppressed Mice

Test	Group (n=6)	0 day	5th day	9th day	13th day
Plt ($10^3/\mu l$)	Normal	1602.5±125.52	1643.2±155.63	1639.5±191.27	1630.7±224.39
	Control	1599.38±127.09	953.5±52.11	780.2±86.92	947.2±199.92
	HPH (200 mg/kg)	1616.88±81.83	1091.0±67.141***	1229.7±182.26***	1296.3±300.82**
	HPH (1000 mg/kg)	1601.13±137.17	1041.5±60.66**	1038.5±143.47***	1395.7±313.73**
	G-CSF (100ul/kg)	1610.88±97.32	1086.5±52.03***	934.83±59.6***	1242.8±222.06**

ICR mice were intraperitoneally injected with 5-FU (200 mg/kg). Two days after administered with HPH (200 mg/kg, 1000 mg/kg) and G-CSF (100ul/kg). Hematologic parameters were monitored on 0st, 5th, 9th and 13th day after 5-FU treatment. Each data represents the mean±S.D. of 6 mice. Statistical significance was compared with control group by t-test. (*: not significant, **: $p<0.05$, ***: $p<0.001$)

2. Histological analysis of BM

Histological data demonstrated that the control group had low density of BM cellularity with large vacuoles (Photo 2). On the other hand G-CSF (100 ul/kg) treated groups had high density of BM cellularity without large vacuoles (Photo. 5). And then there is no difference between control and HPH treated groups in the vacuoles of BM. But HPH treated groups had higher density of BM cellularity than the control group in dose-dependent manner (Photo. 3-4).These results imply that HPH and G-CSF groups have significant effect on hematopoiesis compared with control group.

Table 5. Immunoassay of Cytokine Related with Hematopoiesis

Group (n=6)	IL-3 (pg/ml)	GM-CSF (pg/ml)	TPO (pg/ml)
Normal	16.92±3.73	97.00±81.28	1846.59±447.39
Control	13.98±0.44	46.19±37.83	2833.81±724.09
HPH (200 mg/kg)	15.54±1.92**	108.00±28.04**	1910.51±503.58**
HPH (1000 mg/kg)	14.08±1.11*	89.33±80.29*	1755.68±519.68**
G-CSF (100ul/kg)	16.80±2.13**	59.33±39.16*	2914.77±1024.50*

ICR mice were intraperitoneally injected with 5-FU (200 mg/kg). After this ICR mice were administered with HPH (200 mg/kg, 1000 mg/kg) and G-CSF (100 units/kg). Immunoassay of cytokines were measured on 5th day after 5FU treatment.Each data represents the mean ± S.D. Statistical significance was compared with control group by t-test. (*: not significant, **: $p<0.05$)

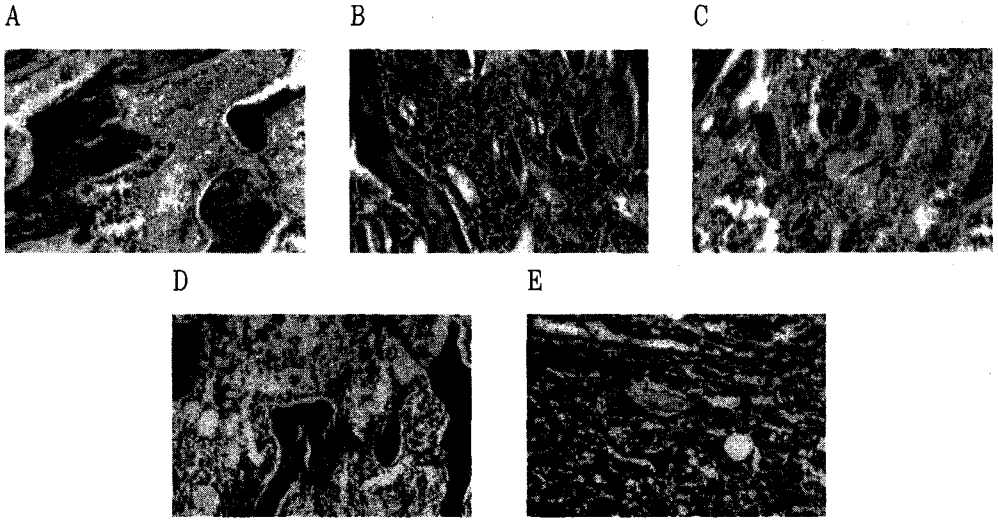

Figure 1. Histological analysis of bone marrow. The bone marrow tissues were isolated from the 5-FU plus HPH and G-CSF treated mice. The isolated tissues were fixed, dissected and stained with hematoxylin and eosin. The image was viewed at 100X magnification.

3. Immunoassay of cytokine

Serum IL-3, GM-CSF and TPO level were measured on 5th day after 5-FU treatment in ICR mice. All of treated groups were slightly increased in IL-3 compared with control group, but there was no statistical significance in HPH (1000 mg/kg) treated group. In GM-CSF levels, HPH (200 mg/kg) treated group showed a marked increase compared with control group. While, TPO levels in the serum were decreased in HPH groups. The results were described in table 5.

DISCUSSION

HPH is derived from human placenta. HPH has been used as a medicinal stuff for a ling time in Oriental Medicine and it has been shown to have clinical efficacy.We previously demonstrated that administration of HPH into normal mice significantly showed a protective effect on the BM failure induced by cyclophosphamide (CY) and irradiation[21]. This study also showed that oral administration of HPH significantly inhibited a reduction of leukocytes after 5-FU treatment and accelerated recovery from 5-FU induced leukopenia.

G-CSF is routinely used to hasten recovery from chemotherapy-induced neutropenia[22].To study the effect of HPH compared with G-CSF on the peripheral blood and hematopoietic stem cell, we treated mice with 5-FU(200 mg/kg) and subsequently administered with HPH(200 mg/kg, 1,000 mg/kg) and G-CSF(100 μl/kg) for 11 consecutive days. HPH and G-CSF treatment groups showed a significant increase in cell numbers of WBC and platelet compared with

control group. It is notable that HPH showed a robust augmentation in WBC compared with G-CSF. These results indicated that HPH increased the cell types which involve in primary hematopoiesis mechanism.

Hematopoiesis is mainly occurring in BM.To verify that the increase in WBC and platelet in blood from the HPH-treated mice was due to an activation of hematopoiesis in the BM, histological analysis and cell composition of BM was carried out. 5-FU treatment results in low density of BM cellularity with large vacuoles. However, G-CSF and HPH treatment produced the higher density of BM. Especially, G-CSF treatment inhibited the formation of large vacuole. In cell composition of BM, 5-FU treatment showed marked decrease of the ratio of leucocyte to erythrocyte. Meanwhile HPH or G-CSF treatment increased the ratio of leucocyte to the erythrocyte. These results imply that HPH treatment activate BM functions in myelosuppression.

To examine actions of HPH on microenvironment of BM, we measured immunoassay and gene expression of cytokine related with hematopoiesis. In the immunoassay of cytokine related with hematopoiesis, all of treated groups were slightly increased in IL-3 compared with control group.Especially, HPH (200 mg/kg) treated group showed a marked incerase in GM-CSF levels compared with control group. However, TPO level in the serum was decreased in HPH groups. IL-3 has also been known as multi-functioning colony-stimulating factor (CSF), stimulating myeloerythroid progenitor cells to differentiate mature myeloid, megakaryocytic and erythroid progenitor cells. IL-3 is required to induce the stem cell to enter into one of five lineages (megakaryocytes, erythrocytes, basophils, neutrophils or eosinophils) and is also required during further differentiation of the granulocytes and monocytes[19, 23]. GM-CSF has an ability to stimulate proliferation and maturation of granulocytes and macrophage myeloid cells.This cytokine also stimulates various functional activities of leukocytes including cytotoxicity of granulocytes and macrophages, phagocytic activity and degranulation of neutrophils[8, 24]. These results imply that HPH stimulates various functional activities of leukocytes.

In previous studies, it is reported that BM has a limited reserve capacity. For example, G-CSF combined with stem cell-damaging cytotoxic agents, results in enhanced stem cell damage and loss of marrow induced by extensive proliferative stress to the BM. This experimental model was designed to investigate short-term repopulating and enhancing hematopoietic ability of BM. We need to study long-term hematopoietic effect of HPH in future[22, 29-30].

Taken together, we demonstrated that HPH functioned in an enhancement of neutrophils and platelet in the BM as well as peripheral blood in the chemically damaged mice. The effect of HPH might be resulted from the regulation of the cytokine expression which is responsible for hematopoiesis such as GM-CSF, IL-3, and TPO. These results suggest that HPH is good candidates for new drugs or therapeutics on chemotherapy-associated myelosuppression. More needs to be learned about their mechanisms of action and therapeutic potential and more clinical trials should be expected.

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