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원 저

Investigations into the immunomodulatory activity of *Ulmus davidiana Planch* extracts

Lee Eon-do, Yoon Jong-hwa, Lee Seung-deok, Kim Kap-sung and Kim Kyung-ho

Department of Acupuncture & Moxibustion, Graduate School of Oriental Medicine, Dongguk University

Abstract

Objective : Although the effect of Ulmus davidiana Planch (UD) extracts on collagen-induced-arthritis (CIA) and bone metabolism has been studies, research on its effect on human immunomodulatory activity is further a due. The objective of the present study was to investigate the immunomodulatory activity of UD on cellular and humoral immunity.

Methods : Oral administration of the ethanolic and water extracts of UD, at doses of 20, 100 and 200 ㎎/㎏ in mice, dose dependently potentiated the delayed type hypersensitivity reaction induced both by sheep red blood cells (SRBC) and oxazolone.

Results : It significantly enhanced the production of circulating antibody titre in response to SRBC in mice. Extracts of UD failed to show any effect on macrophage phagocytosis. Chronic administration of UD extracts significantly ameliorated the total white blood cell count and also restored the myelosuppressive effects induced by cyclophosphamide.

Conclusion : The present investigation reveals that UD extracts possesses immunomodulatory activity.

Key words : Ulmus davidiana Planch (Ulmaceae) ethanol extract, water extract, Immunomodulation, Cell-mediated immunity, Humoral immunity, Phagocytosis

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· **Corresponding author** : Kim Kyung-ho, Dongguk University, Oriental Hospital, #87-2, Sunae-dong, Bun dang-gu, Sungnam-si, Kyunggi-do
Tel. 82-31-710-3746 E-mail : omdkkh@hanmail.net

Ⅰ. Introduction

We observed from the experiment which precedes, the effect of Ulmus davidiana Planch(UD) herbal acupuncture solution regarding the rheumatoid arthritis or bone metabolic disease mainly. Recently does UD how operate to the immunity function of the human body has interest regarding and it approaches it's informs.

The immune system is involved in the etiology as well as pathophysiologic mechanisms of many diseases. Modulation of the immune responses to alleviate the diseases has been of interest for many years[1].

Korean medicinal plants are a rich source of substances, which are claimed to induce paraimmunity, the non specific immunomodulation of essentially granulocytes, macrophages, natural killer cells and complement functions[2]. In the Korean medicine, Korean traditional system of medicine, lays emphasis on promotion of health - a concept of strengthening host defenses against different diseases. These plants, labelled as 'Korean medicine', have been endowed with multiple properties like delaying the onset of senescence and improving mental functions by strengthening the psycho neuro immune axis[3]. The isolation, purification and chemical characterisation of the immunoactive moieties have been carried out in some of these plant.

Ulmus davidiana Planch is a deciduous tree, which is widely distributed in Korea. The barks of the stem and the root of the plant have been used in oriental traditional medicine for the treatment of oedema, mastitis, gastric cancer, and inflammation[4-5]. UD is also known for their functions in maintaining or assisting blood circulation. With these background, UD water extract has been developed on the basis of the known function of the herb, as described in the literature of traditional Korean and Chinese medicine[6-7].

Recently, although UD has been used for protection against degeneration of cartilage and regeneration of damaged tissue, little is still known about the mode of action of this traditional medication on RA. Therefore, as a part of our search for new biologically active substances from traditional medicines, we have evaluated whether extracts of UD could modulate immunosuppression on the induction of cyclophosphamide in mice.

During the primary course of evaluation of immunomodulatory activity of native medicinal plants to identify plant-based immunomodulators, the ethanolic and water extract of the UD was found to stimulate the immune system. The present study was aimed at exploring the influence of UD extracts on humoral and cell mediated immune responses and phagocytic function of the cells of the reticuloendothelial system.

Ⅱ. Materials and methods

1. Material and Preparation of Plant Extract

Drugs used were cyclophosphamide (Sigma CO., USA) and D-penicillamine (Nakarai Pure Chemicals, Kyoto, Japan). For dosing, the drugs were dissolved in saline.

The powder of UD was obtained from the local market and authenticated based on its microscopic and macroscopic characteristics. A voucher specimen (UD-1) has been deposited in the herbarium of Kyungju College of Oriental Medicine, Kyungju, Korea. For the ethanolic extracts, the root powder was

exhaustively extracted using 95% ethanol (1000 ml) in a Soxhlet extractor. The extract was concentrated under reduced pressure and low temperature (40°C) to yield a syrupy mass (yield 12.8%).

An aqueous suspension of the extract referred to as UD ethanolic extract (UDEE) prepared in 0.1% sodium carboxymethylcellulose was used for experimental studies.

The vehicle alone served as control. Previous phytochemical screening of the roots of the plant has shown presence of a disubstituted tetrahydrofuran, caffeic acid-derivatives and steroids. Water extracts of UD (UDWE) was obtained by previous paper[8].

2. Animals

Inbred Swiss albino mice weight between 18 and 25 g were used. The animals were housed under standard conditions of temperature (23±1°C), relative humidity (55±10%), 12/12 h light/dark cycles and fed with standard pellet diet (Daehan Experimental Animals Ltd, Seoul, Korea) and tap water ad libitum.

3. Antigens and DTH Reaction using SRBC and Oxazolone as Antigens

Sheep red blood cells (SRBC) were obtained from Sigma Ltd(St. Louis, USA) SRBC, collected in Alsever's solution, were washed three times in large volumes of pyrogen-free, sterile saline. Oxazolone was purchased from Sigma Chemical Co.(St. Louis. USA)

For SRBC as an antigen, the method described by Doherty[9] was used. Mice of either sex were divided into six groups of six each. UDEE (20, 100, 200mg/kg, p.o.), UDWE (10, 20, 40mg/kg, p.o.) and D-penicillamine (20 mg/kg, p.o.) were administered on day 0 and continued till the day of challenge.

The mice were primed with 0.1 ml of SRBC suspension containing 1× 108 cells, i.p., on day 7 and challenged on day 14 with 0.05 ml of 2× 108 SRBC in the right hind foot pad. The contralateral paw received equal volume of saline. The thickness of the foot pad was measured at 0, 22, 24, 28, 48 and 72 hrs after challenge using Mitutoyo Dial Caliper (Mitutoyo manufacturing Company, Japan). The difference in the thickness of the right hind paw and the left hind paw was used as a measure of delayed type hypersensitivity (DTH) reaction.

For oxazolone as an antigen, the method described by Griswold et al.[10] was used. Mice of either sex were divided into six groups of six each.

The treatment schedule was similar to that of SRBC-induced DTH reaction. On day 7, mice were sensitized by applying 0.1 ml of 3% oxazolone in absolute ethanol to the skin of shaved abdomen. They were challenged on day 14 for contact hypersensitivity using 0.1 ml of 3% oxazolone in absolute ethanol by applying it to both sides of the right hind paw, the contralateral paw received equivalent volume of ethanol. The thickness of the foot pad was measured at 0, 22, 24, 28, 48 and 72 hrs as described earlier.

4. Humoral Antibody Response to SRBC

Mice of either sex were divided into five groups of six each. UDEE (20, 100, 200 mg/kg, p.o.) and UDWE (10, 20, 40 mg/kg, p.o.) was administered on day 0 and continued till the day of the experiment. Cyclophosphamide (50 mg/kg, p.o.) was administered 2 days before the experiment. On day 7, the mice were immunised with 0.1 ml of 1× 108 SRBC, i.p. Blood samples were collected from the orbital

plexuses of individual animals on day 14 and the antibody titres were determined using the method described by Puri et al.[11] Briefly, an aliquot (25㎕) of twofold diluted sera in saline was challenged with 25㎕ of 0.1% v/v SRBC suspension in microtitreplates. The plates were incubated at 37°C for 1 h and then observed for hemagglutination. The highest dilution giving hemagglutination was taken as the antibody titre. The antibody titres were expressed in a graded manner, the minimum dilution (1/2) being ranked as 1. The mean ranks of different groups were statistically compared.

5. Macrophage Phagocytosis by Carbon Clearance Method

Mice of either sex were divided into four groups of six each. UDEE (at doses of 20, 100 and 200 ㎎/㎏, p.o.) and UDWE (at doses of 10, 20 and 40 ㎎/㎏, p.o.) was administered 15 days prior to injection of carbon particles. On day 16, mice were injected with 0.1 ㎖ of carbon suspension (Pelikan Tuschea Ink, Germany), i.v., through the tail vein (Biozzi et al.)[12]. Blood samples (25㎕) were collected from the orbital plexuses of individual animals immediately before and at 3, 6, 9 and 12 min after the injection of carbon suspension, lysed with 2 ㎖ of 0.1% glacial acetic acid and the absorbance was measured spectrophotometrically at 675 ㎚ (Atal and Sharma)[13]. The rate of carbon clearance, termed as phagocytic index, was calculated as the slope of time concentration curve.

6. Effect of chronic Administration of UDEE and UDWE on peripheral Blood Count

The method used was as described by Ziauddin et al.[9]. Mice of either sex were divided into four groups of ten each. The animals received UDEE (at doses of 20, 100 and 200 ㎎/㎏, p.o.) and UDWE (at doses of 10, 20 and 40 ㎎/㎏, p.o.) for 15 days. On day 16, blood was collected from orbital plexuses of the individual animals and total WBC, RBC, HCT, MCV and haemoglobin were determined using Erma PC 607 cell counter (Erma Inc., Japan). The differential leukocyte count was performed by fixing the blood smears and staining with Field Stains A and B, and percent neutrophils in each sample was determined.

7. Effects of UDEE and UDWE on cyclophosphamide-induced Immunosuppression

The method used was as described by Ziauddin et al.[14]. Mice were divided into five groups, each containing ten mice. Groups Ⅲ, Ⅳ and Ⅴ received UDEE (at doses of 20, 100 and 200 ㎎/㎏, p.o.) and UDWE (at doses of 10, 20 and 40 ㎎/㎏, p.o.), respectively, for 15 days prior to administration of cyclophosphamide. Groups Ⅱ to Ⅴ received cyclophosphamide at a dose of 30 ㎎/㎏, p.o., for the next 3 days. On day 19, blood was collected from retro-orbital plexuses of individual animals and white blood cell count was determined using Erma PC-607 cell counter (Erma Inc., Japan).

8. Statistical Analysis

The results are presented as Mean±SD. Statistical significance between the groups was analysed by Student's t-test. $P<0.05$ was considered to be statistically significant.

III. Results

1. DTH Reaction using SRBC as an Antigen

UDEE (20, 100 and 200 mg/kg, p.o.) and UDWE (10, 20 and 40 mg/kg, p.o.) produced significant, dose-related increases in DTH reactivity in mice. The edema achieved a peak at 24 h, the percent edema being 37.4% for the control group, after which it subsided. D-Penicillamine produced significant inhibition of edema as compared to control at 24 hrs(Table 1, Fig. 1)(Table 2, Fig. 2).

Table 1. Effects of UDEE (20, 100 and 200 mg/kg, p.o.) on SRBC (1×108 cells, i.p.)-Induced DTH at 22, 24, 28, 48 and 72 hrs.

	Control	Penicillamine (20 mg/kg)	UDEE (20 mg/kg)	UDEE (100 mg/kg)	UDEE (200 mg/kg)
% Edema					
22 h	31.4±2.4	22.5±2.4*	40.5±3.2	50.3±2.5	61.2±3.5*
24 h	32.4±1.5	21.3±2.3	45.4±7.2	54.5±3.3	62.5±3.5*
28 h	31.3±2.6	20.4±2.5*	40.2±4.2	51.3±2.5	50.4±3.5*
48 h	17.5±1.6	15.1±1.6	25.4±2.4	31.5±3.2	32.3±2.6
72 h	6.3±0.3	6.1±0.2	14.5±1.4	15.5±1.6	12.6±1.5

Each value represents the Mean±SD. of six observations.
*P<0.05 (Student's t-test).

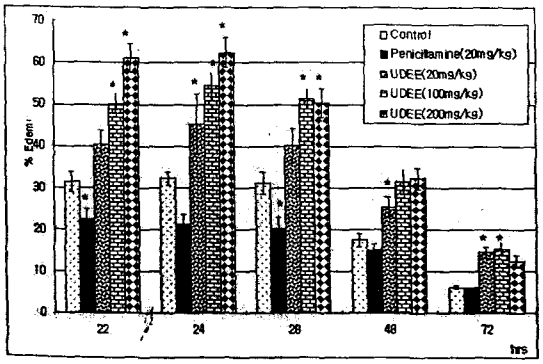

Fig. 1. Effects of UDEE on SRBC-Induced DTH at 22, 24, 28, 48 and 72 hrs.

Table 2. Effects of UDWE (10, 20 and 40 mg/kg, p.o.) on SRBC (1×108 cells, i.p.)-Induced DTH at 22, 24, 28, 48 and 72 hrs.

	Control	Penicillamine (20 mg/kg)	UDWE (10 mg/kg)	UDWE (20 mg/kg)	UDWE (40 mg/kg)
% Edema					
22 h	33.2±5.1	22.4±2.4*	37.4±3.3	43.4±4.2*	52.2±3.5*
24 h	36.3±2.5	25.3±2.5*	45.5±3.6	51.2±3.7*	56.3±3.3*
28 h	33.2±3.5	21.2±1.4*	42.3±4.5	45.3±5.3*	46.3±1.4*
48 h	18.3±2.5	13.5±1.3	24.4±2.2	25.3±2.1	26.4±2.1
72 h	6.0±0.1	6.1±0.2	9.6±.6	11.3±1.1	11.2±1.2

Each value represents the Mean±SD. of six observations.
*P<0.05 (Student's t-test).

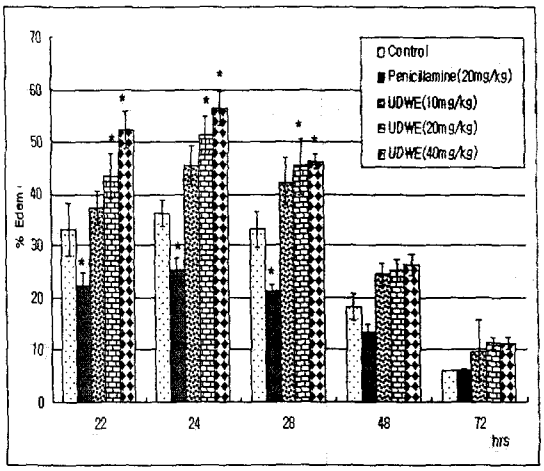

Fig. 2. Effects of UDWE on SRBC-Induced DTH at 22, 24, 28, 48 and 72 hrs.

2. DTH Reaction using Oxazolone as an Antigen

UDEE (20, 100 and 200 mg/kg, p.o.) and UDWE (10, 20 and 40 mg/kg, p.o.) produced significant, dose-related increases in DTH reactivity in mice. The edema achieved a peak at 24 h, the percent edema for the control group being 24.2%, after which it subsided. D-penicillamine produced significant inhibition of edema as compared to control at 24 h(Table 3, Fig. 3)(Table 4, Fig. 4).

Table 3. Effects of UDEE(20, 100 and 200 mg/kg, p.o.) on Oxazolone(0.1ml of 3% oxazolone)-Induced DTH at 22, 24, 28, 48 and 72 hrs.

	Control	Penicillamine (20 mg/kg)	UDEE (20 mg/kg)	UDEE (100 mg/kg)	UDEE (200 mg/kg)
% Edema					
22 h	23.1±2.3	12.3±2.1*	26.6±2.1	32.3±2.3*	44.2±2.2*
24 h	24.5±2.5	14.4±1.5*	27.2±2.5	35.4±3.3*	48.6±3.2*
28 h	20.2±1.2	13.6±1.1*	26.3±2.4	30.3±1.3*	43.6±2.3*
48 h	17.3±1.1	9.5±0.5*	22.4±1.3	18.4±1.3	31.4±2.4
72 h	6.0±0.4	3.3±0.2*	8.2±0.3	6.7±0.5	12.4±1.5

Each value represents the Mean±SD. of six observations.
*P<0.05 (Student's t-test).

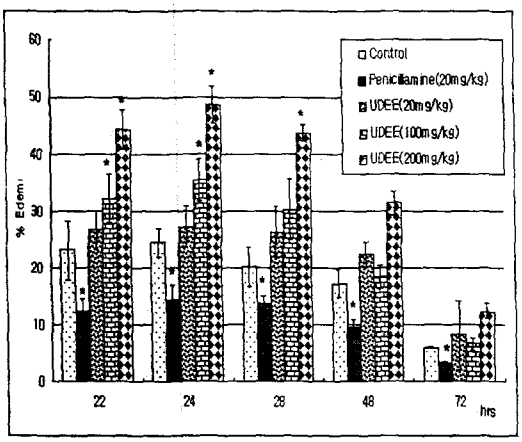

Fig. 3. Effects of UDEE on Oxazolone Induced DTH at 22, 24, 28, 48 and 72 hrs.

Table 4. Effects of UDWE (10, 20 and 40 mg/kg, p.o.) on Oxazolone(0.1ml of 3% oxazolone)- Induced DTH at 22, 24, 28, 48 and 72 hrs.

	Control	Penicillamine (20 mg/kg)	UDWE (10 mg/kg)	UDWE (20 mg/kg)	UDWE (40 mg/kg)
% Edema					
22 h	23.4±2.5	10.3±1.5*	22.3±2.2	24.2±2.2*	41.3±2.4*
24 h	23.6±2.3	12.2±1.4*	24.5±2.2	29.4±2.3*	42.4±3.3*
28 h	20.2±1.5	11.5±1.2*	22.3±1.3	28.2±2.4	38.3±3.2*
48 h	15.6±1.2	8.6±0.6*	18.3±1.7	13.2±1.2	23.4±1.3*
72 h	6.1±0.4	3.3±0.4	3.4±0.3	2.4±0.3	8.2±0.2*

Each value represents the Mean±SD. of six observations.
*P<0.05 (Student's t-test).

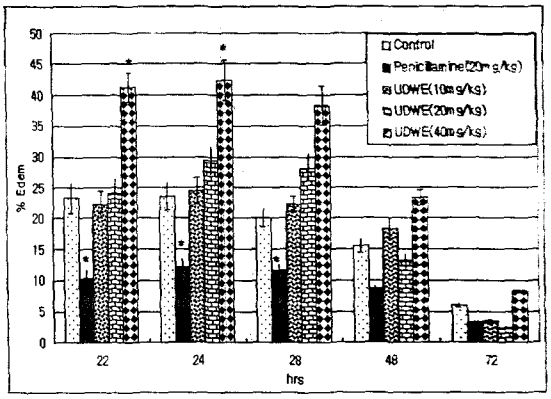

Fig. 4. Effects of UDWE on Oxazolone Induced DTH at 22, 24, 28, 48 and 72 hrs.

3. Humoral Antibody Response to SRBC and Macrophage Phagocytosis by Carbon Clearance Method

A significant, dose-related increase in humoral antibody titres was observed in mice treated with UDEE (20, 100 and 200 mg/kg, p.o.) and UDWE (10, 20 and 40 mg/kg, p.o.). UDWE was more superior than UDEE.

UDEE (20, 100 and 200 mg/kg, p.o.) and UDWE (10, 20 and 40 mg/kg, p.o.) did not show any significant potentiation of the macrophage phagocytic activity as evident by the phagocytic index (Table 5, Fig. 5).

Table 5. Effects of the UDEE and UDWE on Humoral Antibody Titre and Macrophage Phagocytosis

Treatment	Dose (mg/kg, p.o.)	Humoral antibody titre	Phagocytic index
Control		5.4±0.2	0.053±0.03
Cyclophosphamide	50	4.0±0.2*	-
UDEE	20	6.5±0.2	0.052±0.03
UDEE	100	7.4±0.3*	0.052±0.02
UDEE	200	8.3±0.4*	0.051±0.02
UDWE	10	7.7±0.5*	0.062±0.02
UDWE	20	7.9±0.6*	0.063±0.02
UDWE	40	8.5±0.5*	0.052±0.01

Values are expressed as Mean±SD. of six observations.
* P<0.05 as compared to control.

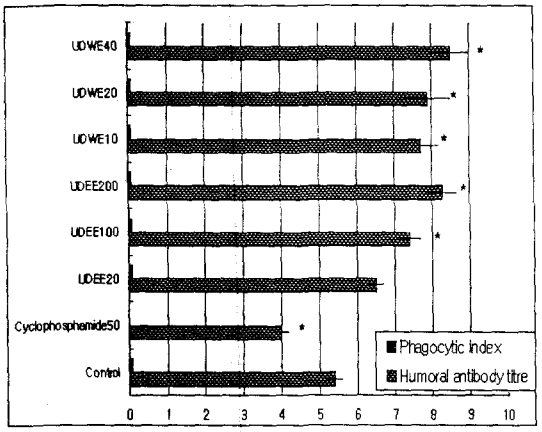

Fig. 5. Effects of the UDEE and UDWE on Humoral Antibody Titre and Macrophage Phagocytosis.

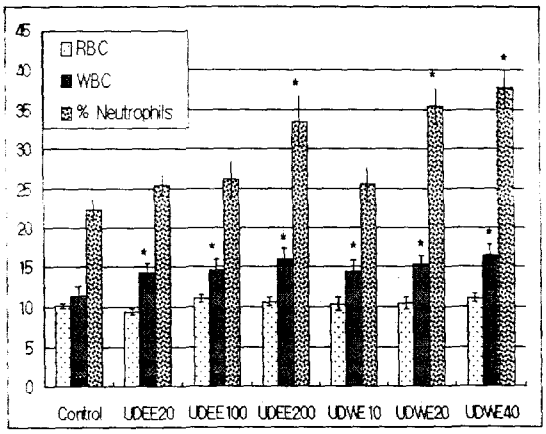

Fig. 6. Effect of Chronic Administration of UDEE and UDWE on Peripheral Blood Count.

4. Effects of UDEE and UDWE on Peripheral Blood Count

A significant, dose-related increase in the WBC count was observed in mice treated with UDEE (20, 100 and 200 mg/kg, p.o.) and UDWE (10, 20 and 40 mg/kg, p.o.) for 15 days.

However, no significant changes were observed in the other haematological parameters (Table 6, Fig. 6).

Table 6. Effects of Chronic Administration of UDEE and UDWE on Peripheral Blood Count

Treatment	Dose (mg/kg, p. o.)	RBC ($x10^6$ cells/$\mu\ell$)	WBC ($x\ 10^3$ cells/$\mu\ell$)	Neutrophils (%)
Control		10.2±0.3	11.4±1.2	22.4±1.3
UDEE	20	9.5±0.4	14.3±1.2*	25.4±1.4
UDEE	100	9.5±0.4	14.7±1.3*	26.2±2.3
UDEE	200	11.1±0.3	16.1±1.4*	33.5±3.2
UDWE	10	10.7±0.5	14.5±1.3*	25.5±2.3
UDWE	20	10.4±0.8	15.4±1.2*	35.4±2.3
UDWE	40	11.1±0.5	16.4±1.4*	37.6±2.5

Values are expressed as Mean±SD. of six observations.
* P<0.05 as compared to control.

5. Effects of UDEE & UDWE on Cyclophosphamide Induced Immunosuppression

Cyclophosphamide at the dose of 30 mg/kg, p.o. caused a significant reduction in the white blood cell count. Combined treatments of cyclophosphamide and UDEE, and UDWE resulted in restorations of bone marrow activity as compared with cyclophosphamide treatment alone (Table 7, Fig. 7).

Table 7. Effects of Chronic Administration of UDEE and UDWE on Cyclophosphamide (30 mg/kg, p.o.) Induced Myelosuppression.

Treatment	Dose (mg/kg, p. o.)	RBC ($x\ 10^6$ cells/$\mu\ell$)	WBC ($x\ 10^3$ cells/$\mu\ell$)	Neutrophils (%)
Control		10.2±1.1	12.3±1.3	18.5±1.3
CY		10.1±0.6	5.9±0.3*	13.2±1.2*
UDEE+CY	20	9.5±0.6	5.5±1.2*	15.4±2.1
UDEE+CY	100	10.5±0.6	8.6±1.2#	18.4±1.4
UDEE+CY	200	9.6±0.5	8.4±1.1#	17.9±1.4
UDWE+CY	10	10.3±1.2	7.4±1.1#	18.2±1.2
UDWE+CY	20	10.3±1.3	8.4±1.2#	19.4±1.3
UDWE+CY	40	10.4±1.2	8.3±1.6#	19.5±1.5

CY: Cyclophosphamide control.
* P<0.05 as compared to control. # P<0.05 as compared to cyclophosphamide control. Values are expressed as Mean±SD. of six observations.

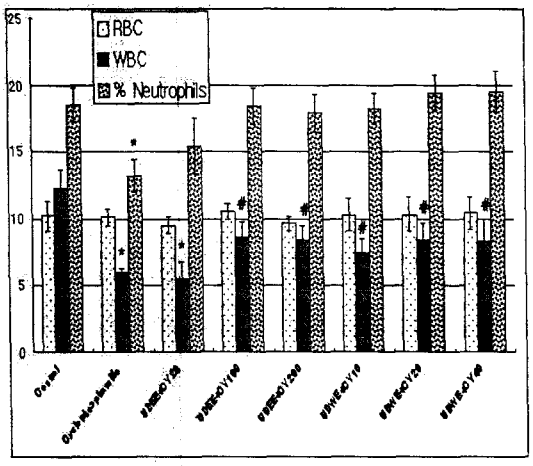

Fig. 7. Effects of Chronic Administration of UDEE and UDWE on Cyclophosphamide (30 mg/kg, p.o.) Induced Myelosuppression.

Recovery period observations indicated that cyclophosphamide induced immunosuppression was not restored to normal, even after discontinuous use of the cyclophosphamide. It was observed that the animals treated with UD showed more normal activity as compared to cyclophosphamide treated group.

Ⅳ. Discussion & Conclusion

Delayed type hypersensitivity (DTH) is a part of the process of graft rejection, tumour immunity, and most important, immunity to many intracellular infectious microorganisms, especially those causing chronic diseases such as tuberculosis[15].

DTH requires the specific recognition of a given antigen by activated T lymphocytes, which subsequently proliferate and release cytokines. These in turn increase vascular permeability, induce vasodilatation, macrophage accumulation[16] and activation, promoting increased phagocytic activity and increased concentrations of lytic enzymes for more effective killing[17].

One of the most potent adjuvants known for inducing T cell immune responses is Freund complete adjuvant, which contains pieces of heat-killed mycobacteria[18].

The immune system can be broadly divided into 2 components, the innate immune system and the adaptive immune system. Innate immunity refers to cellular components include monocytes and macrophages, neutrophils, eosinophils, natural killer(NK) cells, and the complement cascade. Several animal models have demonstrated that specific activation of innate effectors(eg, monocytes, macrophages, eosinophils) can induce potent killing of tumor cells[19].

In the present study, SRBC and oxazolone, a chemical sensitizer which in combination with skin proteins acquires antigenicity[20], were used to elicit contact hypersensitivity reaction in mice. It was found that UDEE and UDWE dose-dependently potentiated the DTH reaction induced both by SRBC and oxazolone. Increase in DTH reaction in mice in response to thymus-dependent antigen revealed the stimulatory effect of UDEE and UDWE on T lymphocytes and accessory cell types required for the expression of reaction[21].

The humoral immunity involves interaction of B cells with the antigen and their subsequent proliferation and differentiation into antibody-secreting plasma cells.

Antibody functions as the effector of the humoral response by binding to antigen and neutralizing it or facilitating its elimination by cross-linking to form clusters that are more readily ingested by phagocytic cells.

To evaluate the effect of UD on humoral response, its influence was tested on sheep erythrocyte-specific haemagglutination antibody titre in mice. Cyclophosphamide at a dose of 50 mg/kg, p.o., showed significant inhibition in

antibody titre response, while UDEE and UDWE were found to significantly enhance the production of circulating antibody titre. This indicates the enhanced responsiveness of macrophages and T and B lymphocyte subsets involved in antibody synthesis[22].

The role of phagocytosis is primarily the removal of microorganisms and foreign bodies, but also the elimination of dead or injured cells. Phagocytic defects are associated with varied pathological conditions in humans[23].

In view of the pivotal role played by the macrophages in coordinating the processing and presentation of antigen to B-cells, UDEE and UDWE were evaluated for its effect on macrophage phagocytic activity. When the carbon particles are injected intravenously, the rate of clearance of carbon from blood by macrophage is governed by an exponential equation. This seems to be the general way in which inert particulate matter is cleared from the blood. In this study, however, UDEE and UDWE failed to show any effect on macrophage phagocytosis.

UD is an extract developed to have therapeutic effects in inflammatory diseases involving cartilage destruction, such as RA. According to published work that is well accepted by the traditional medicine community, UD was formulated to facilitate blood circulation as well as to reduce inflammatory activity. The UD have been used for hundreds of years in this asian region, and their safety and efficacy are well established through a long history of human use, but their use still lacks scientific support[7,24].

Although the barks of UD stem and root have been used in oriental traditional medicine for inflammatory diseases, the action mechanisms of this species are not nearly understood. It may be important to understand how this plant extract performs investigations into the immunomodulatory activity of UD extracts action in vivo.

Since UDEE and UDWE augmented the circulating antibody titre, it was thought worthwhile to evaluate its effect on peripheral blood count and cyclophosphamide-induced immunosuppression. Chronic administration of UDEE and UDWE significantly ameliorated the total white blood cell count and also restored the myelosuppressive effects induced by cyclophosphamide. The present investigation suggests that UD may stimulate both the cellular and the humoral immunity. Further studies to elucidate the exact immunostimulatory mechanism of UD are in progress.

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