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Ulmus macrocarpa Hance Reduces Cyclophosphamide-induced Toxicity in Mouse Liver

Deok Won Kim and Kyung Tae Chung*

Department of Clinical Laboratory Science Graduate School, Dong-Eui University, Busan 47340, Korea

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Cyclophosphamide (CP) is widely used in cancer and lymphoma treatments and as an immunosuppressant drug. CP is a DNA alkylating agent that metabolizes into 4-hydrocyclophosphamide (4H-CYP) and aldophosphamide in hepatocytes. However, its metabolites cause DNA synthesis disorder, leading to apoptosis and toxic side effects. The development of technology to minimize this side effect is essential to improve CP's clinical application. Various bioactive compounds have been reported to have anti-cancer and antioxidant functions and preventive or therapeutic roles in metabolic diseases. Many researchers have attempted to minimize the side effects and improve the efficacy of these drugs together with the use of bioactive compounds. *Ulmus macrocarpa* Hance has been used for the treatment of edema, mastitis, stomach pain, tumors, cystitis, and other inflammatory diseases. The aim of this study was to investigate at the histological level the protective function of *U. macrocarpa* Hance against CP's side effects and any potential toxic effect of *U. macrocarpa* Hance in the liver and kidney. Water extracts of *U. macrocarpa* Hance reduced CP-induced toxicity and did not induce any histological damage in the liver and kidney. Therefore, *U. macrocarpa* Hance would be applicable in the pharmaceutical industry.

Key words : Cyclophosphamide, histological damage, kidney, liver, *Ulmus macrocarpa*

Introduction

Cyclophosphamide (CP) is a DNA alkylating agent. When administered, CP is metabolized into 4-hydrocyclophosphamide (4H-CYP) and aldophosphamide by the cytochrome P-450 enzyme present in the hepatocytes. Aldophosphamide is further decomposed into phosphoramide mustard and acrolein, which are active metabolites. CP is widely used in cancer and lymphoma treatments as well as an immunosuppressant drug [1, 11]. However, the clinical use of CP has been limited due to its ability to damage normal tissues which usually resulted in multiple organ toxicity mainly in the heart, testes, urinary bladder, and liver [2, 20, 26].

There are many reports on various bioactive compounds that have anti-cancer, antioxidant functions, and preventive and/or therapeutic roles in metabolic diseases [5, 24, 25]. In other words, simultaneously, bioactive compounds protect organs against harmful chemicals as well as other cell stress factors. Catechin is one of the most well-known bioactive compounds and has been reported for biological function of anti-inflammation and anti-oxidation on cancer, cardiovascular disease, and metabolic diseases such as diabetes [9, 19]. Resveratrol is a strong anti-oxidant bioactive compound suppressing NADPH oxidase-mediated production of ROS [27]. Furthermore, resveratrol was reported for its anti-aging effects observed in lower organisms [3]. Quercetin is enriched in onion peel and can be ingested as a part of common diets and has anti-hypertensive actions mimicking verapamil, a Ca^2+ channel blocker that reduces blood pressure [16].

Recently, diverse biological functions of *Ulmus macrocarpa* Hance such as antioxidant, antihypertensive, anti-cancer, and anti-thrombotic activity have been studied [13, 18, 28]. *Ulmus macrocarpa* Hance is a deciduous tree (Ulmaceae) native to Korea [23]. In traditional medicine, it has been used for the treatment of edema, mastitis, stomach pain, tumors, cystitis as well as other inflammatory diseases [12]. Although major bioactive compounds of *U. macrocarpa* Hance have not been defined well, its reported biological function must depend on bioactive compounds. For clinical use, when administered orally, *U. macrocarpa* Hance should not cause any adverse effects on any organ. All compounds are

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***Corresponding author**
Tel : +82-51-890-2681, Fax : +82-51-890-2622
E-mail : kchung@deu.ac.kr

metabolized in the liver and excreted through the kidney.

In this study, we investigated any potential toxic effect at histological level of *U. macrocarpa* Hance in the liver and the kidney as well as protection function of *Ulmus macrocarpa* Hance against CP which causes side effects on many organs.

Materials and Methods

Experimental animals

12 weeks old male BALB/c mice (23±2 g) were purchased from Samtaco Bio Korea (Osan, Korea). These animals were kept under standard conditions with temperature maintaining 24±1℃, humidity 55±5%, and 12 hr dark-light cycle. Food and water were freely accessible. All experimental procedures were followed by the guidelines of the Institutional Animal Care and Use Committee of Dong-Eui University (R2014-017).

Preparation of *Ulmus macrocarpa* Hance water extract

Cortex of *Ulmus macrocarpa* Hance was purchased from Dae-Han herbal medicine Inc. (Busan, Korea). Water extraction of the cortex of *U. macrocarpa* Hance was produced by heating at 95℃ for 6 hr and then filtered with an 80 mesh filter. The filtered extract was concentrated at 75℃ for 2 hr, and lyophilized at -45℃. The *U. macrocarpa* Hance water extract (UMWE) was dissolved in sterilized water before the experiment [15].

Experimental design

The mice were divided into six groups (n=6); Normal control, CP administration, CP+UMWE 100 mg/kg, CP+UMWE 200 mg/kg, UMWE 100 mg/kg, UMWE 200 mg/kg. The mice of test groups were orally administered with UMWE for 14 days and the same amount of sterilized water was orally administered to the control group. Intraperitoneal injection of a dose of 120 mg/kg of cyclophosphamide (Sigma-Aldrich, USA) was administered to the test groups on the 13th day. All animals did not fast until the day of sacrifice.

Organ to body weight ratio

The liver and kidneys were dissected out from the sacrificed animals and the weights of each organ were measured. The organ body weight ratio was calculated by the following formula [4].

$$\text{Organ to body weight ratio} = \frac{\text{Organ weight (mg)}}{\text{Body weight (g)}} \times 100$$

Histological analysis

The liver and kidney were removed and fixed in 10% neutral buffered formalin for 24 hr. The fixed tissues were dehydrated with a tissue processor (LEICA TP 1020, Leica Biosystems, Germany) and subjected to a clear, paraffin infiltration process. Subsequently, the organs embedded in paraffin wax using a paraffin embedding station (Tissue-Tek® TEC ™ 5, Sakura, United States) and slides with a 3 μm section were cut using a microtome (LEICA RM 2235, Leica Biosystems, Germany) and stained with hematoxylin and eosin (H&E). For DAPI staining, tissue sections were deparaffinized and stained with DAPI. The slides were examined under a fluorescence microscope (Axio Scope A1, Carl Zeiss, Germany). The number of hepatocyte nuclei excluding kupffer cells were counted in four corner squares of 25×10^4 pixel^2 of a field of view.

Statistical analysis

Data were analyzed with GraphPad Prism 5 (GraphPad Software, United States). One-way ANOVA and Bonferroni post-test were used to compare multiple groups at significance level $p<0.05$. Results were expressed as mean ± standard deviation.

Results and Discussion

Body weight and organ weight are important indices in toxicity investigation of drugs and natural compounds [10]. In this case ratio of organ weight to body weight (organ weight index) is used to judge toxicity of any compound and especially for liver, organ index weight is required [17, 22]. According to the previous reports, decrease in body weight generally indicates toxicity of test materials [10]. To be consistent with previous reports, single administration of CP in this study showed decrease in body weight which is indicative of toxic effect. In body weight analysis, decrease of CP group's body weight was statistically significant when compared with normal control group's body weight (Table 1). Not only CP group but also body weight of CP+UMWE 100 and CP+UMWE200 groups was decreased. However, CP+UMWE200 group only showed statistical significance. Similarly, the liver weight of CP+UMWE200 group was also decreased. In order to justify decrease of liver weight of CP+

Table 1. Body weight and organ weight of each experimental group (n=6)

Parameter[†]	Normal control	CP	UMWE100+CP	UMWE200+CP	UMWE100	UMWE200
Terminal body weight (g)	25.90±1.45	24.08±1.06*	24.90±0.65	24.10±0.82*	25.28±2.16	25.80±0.91
Organ weight (g)						
Liver	1.270±0.239	1.090±0.212	1.092±0.072	1.012±0.152*	1.162±0.292	1.136±0.183
Kidneys	0.358±0.038	0.321±0.064	0.344±0.063	0.324±0.021	0.354±0.045	0.354±0.035
Organ to body weight ratio (mg/g)						
Liver	48.800±7.101	45.060±7.333	43.850±2.757	41.870±5.009	45.520±8.282	43.870±5.556
Kidneys	13.800±0.816	13.260±2.200	13.780±2.370	13.440±0.699	14.010±1.566	13.710±1.071

[†] Multiple groups of each parameter were analyzed by one-way ANOVA and Bonferroni post-test using GraphPad Prism 5.
*indicates statistically significant difference in groups.
Statistical significance means $p<0.05$.

UMWE200 group organ to body weight ratio was compared. When organ indices were compared there was no statistically significant difference among all groups, which indicates liver weight was proportionally decreased with body weight in CP+UMWE200 group due to CP, not to UMWE intake. Both UMWE100 and UMWE200 groups did not show any decrease in body weight as well as in organ index, suggesting that UMWE would not be toxic. Data of UMWE100 and UMWE200 groups were supporting this interpretation (Table 1). Body weight of UMWE200 group was 25.80±0.91 and liver weight was 1.136±0.0183, which is no statistically significant differences in body and liver weight of normal group.

Hematoxylin and Eosin (H&E) stain is the basic histological staining for a general assessment of cell and tissue morphology. Cellular nucleus shows blue-purple and cytoplasm and the cartilage matrix pinkish red by H&E staining, but neutrally charged molecules such as glycogen does not stain leaving clear areas. When the mouse is fasting , the entire area of cytoplasm of hepatocyte is stained without clear areas by H&E staining because of no or weak accumulation of glycogen. However, when the mouse is fed normally, the cytoplasm of hepatocyte shows clear areas by H&E staining because accumulated glycogen area in cytoplasm is partially unstained.

All mice of each group had not fasted during the experimental period. Hepatocytes of the normal control group showed a typical H&E stained pattern with obvious glycogen accumulation (Fig. 1). In contrast, H&E-stained hepatocytes of the CP group did not show glycogen accumulation. However, hepatocytes of both CP+UMWE100 and CP+UMWE200 groups showed glycogen accumulation but not as much as the amount of glycogen in normal group's hepatocytes. These data suggested that CP's toxicity was alleviated by UMWE intake. Hepatocytes of both UMWE100 and UMWE200 groups showed glycogen accumulation as

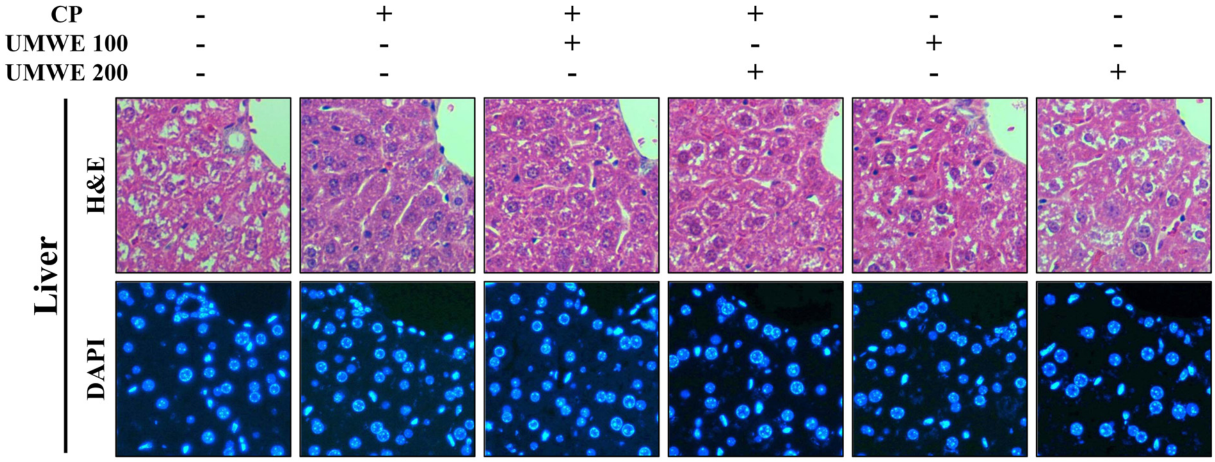

Fig. 1. Histological analysis of liver. Top panel: H&E-stained liver, bottom panel: DAPI-stained liver (x400). Open triangle indicates glycogen accumulation.

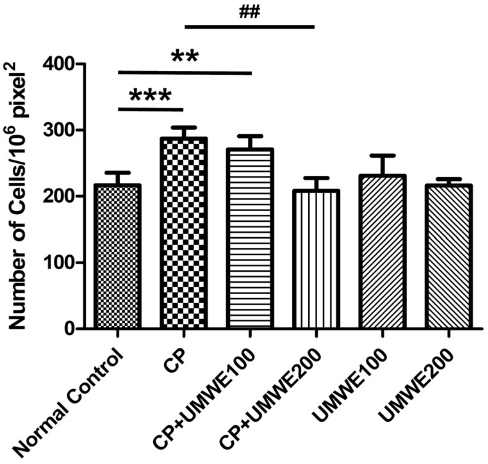

Fig. 2. Number of hepatocytes. DAPI-stained nuclei were counted in four corner squares of field of view. One square was $25x10^4$ pixel^2. All groups were analyzed by one-way ANOVA and Bonferroni post-test using GraphPad Prism 5. *: statistical difference compared with normal control, #: statistical difference compared with CP. *** $p<0.001$, **$p<0.01$, ##$p<0.01$

much as the amount of glycogen in normal group's hepatocytes . Based on these data CP seemed to cause a reduced intake of food which explains loss of body weight and no or less glycogen accumulation in CP-treated groups. On the other hand, UMWE seemed not to cause food take interference. We also analyzed nuclear change with DAPI staining for apoptosis caused by toxicity of CP or UMWE. No obvious apoptotic nucleus was identified (Fig. 1). One difference was density of nucleus which varied among the groups. Number of nuclei was counted to quantify cell numbers in the same size of area. Nuclei in four corner squares of 250,000 pixel^2 were counted with three different slides of each group and statistically analyzed. When compared with the normal group the number of cells of the CP group increased by 32.7% and the number of cells of CP+ UMWE100 also increased by 25.1%. However, the number of cells of CP+ UMWE200 was 27.5% less than that of CP group and remained similar to that of normal group (Fig. 2). These data indicate that volume of hepatocytes of CP group became smaller due to no glycogen accumulation caused by CP toxicity, therefore, cell population increased within the area. It appears that UMWE reduced CP toxicity in both CP+UMWE 100 and CP+UMWE200 groups. Both UMWE100 and UMWE 200 groups without CP maintained as much as normal cell populations, which is again consistent with H&E stained pattern results.

The kidneys are responsible to maintain chemical composition of cells by regulating the amount of water, electrolytes as well as many other molecules. Many drugs including anticancer drugs including cyclophosphamide have been reported to cause renal toxicity [21]. Susceptibility of the organ is due to receiving 20~25% of resting cardiac output, exposure to a higher concentration of drugs during filtration, increased intracellular concentrations of drugs via transporters, and high energy requirement of the tubules [8]. Recently, natural compounds are increasingly reported for treatment use of kidney diseases [14]. Natural compounds from medicinal plants have shown protective activity against nephrotoxicity. However, there is more likely no known study of *U. macrocarpa* effect on kidney under cyclophosphamide administration. Our histological data of the kidney showed that structures of the glomerulus, glomerular capsule, and renal tubular cells were not altered in all groups (Fig. 3). One of the reasons would be a single administration in

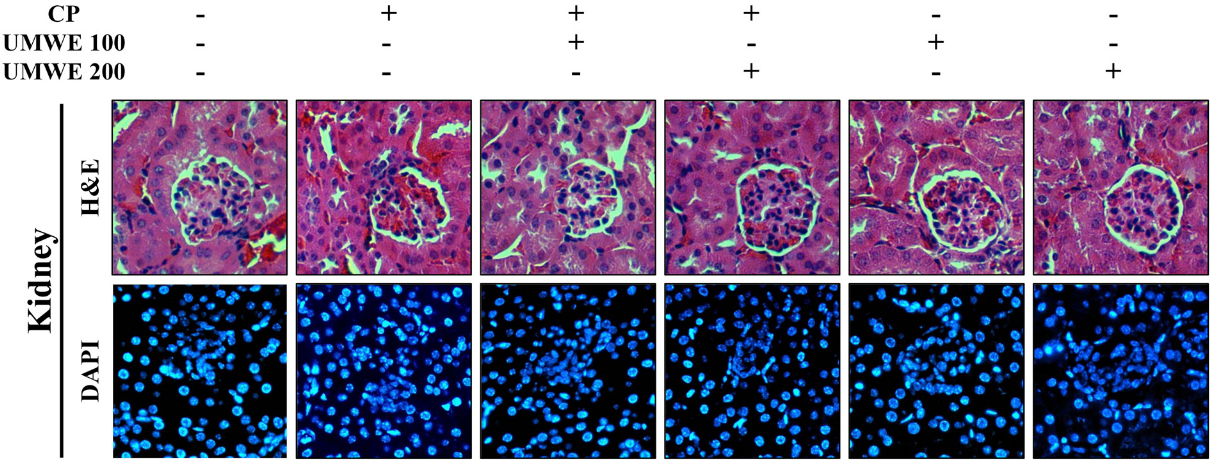

Fig. 3. Histological analysis of kidney. Top panel: H&E-stained kidney, bottom panel: DAPI-stained kidney (×400).

this study. Kidney damage by cyclophosphamide has been reported by multiple administrations during a certain experimental period. Ei-Shabrawy, *et al*, showed tubular and glomerular distortion in the kidney with 6 times administrations during 3 weeks [7]. On the other hand, UMWE did neither cause any histological damage in the kidney by both 100 and 200 mg/kg concentrations, which suggests that UMWE does not have any toxic effect on the kidney after two weeks of a feeding period.

In summary, our data showed that single administration of CP caused histological change in the liver, and two weeks of UMWE feeding before CP administration reduced Cyclophosphamide-Induced toxicity and maintained histological structure close to the normal condition of the liver. Furthermore, UMWE by itself did not show any histological structure change of the liver. Two weeks of lab mouse is almost equivalent to one and half years of human lifespan [6]. Therefore, long-term intake of UMWE may not cause any adverse effect particularly in human liver and regular intake of UMWE would be applicable for nutraceutical tablets. No obvious histological change in kidney was identified by either CP or UMWE. However, this study did not present biochemical data of CT toxicity and UMWE effect which may link direct or indirect action against each other. Bichemical investigation with single and multiple administration of CP requires to better understand more precise beneficial roles of UMWE against CP.

The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : Cyclophosphamide가 유발한 간 조직변화에 대한 느릅나무 열수추출물의 완화 효과

김덕원 · 정경태*
(동의대학교 대학원 임상병리학과)

Cyclophosphamide (CP)는 면역 억제제 뿐만 아니라 암 및 림프종 등의 치료에 널리 사용된다. CP는 DNA 알킬화제로서, 간세포에서 대사되어 4-hydrocyclophosphamide (4H-CYP)와 aldophosphamide로 분리된다. *Ulmus macrocarpa* Hance는 부종, 유방염, 종양 및 기타 염증성 질환에 사용되어 왔다. 이 연구의 목적은 CP의 부작용에 대하여 *U. macrocarpa* Hance 열수 추출물이 조직학적 수준에서 CP의 부작용에 대한 간과 신장의 보호 기능과 *U. macrocarpa* Hance 자체의 잠재적 독성 영향을 조사하고자 하였다. 마우스 모델을 사용하여 헤마톡실린 및 에오신(H&E) 염색과 DAPI 염색으로 간과 신장을 조직학적으로 분석하였다. CP 처리한 마우스에서 간세포의 형태는 글리코겐 축적을 나타내지 않았고, 세포 밀도도 감소하였다. 그러나 UMWE+CP 처리군에서는 간세포의 형태와 세포 밀도는 정상 간세포 패턴과 유사하였다. 또한, UMWE으로만 처리한 마우스에서도 간세포의 형태와 세포 밀도는 정상 간세포와 유사했다. 신장의 경우에는 정상 마우스와 비교했을 때 H&E 염색으로는 CP 또는 UMWE 처리된 마우스의 신장에서 명백한 차이를 나타내지 않았다. 즉, *U. macrocarpa* Hance의 열수추출물은 간과 신장에 아무런 영향을 유발하지 않으면서 CP가 유발한 독성을 감소시키는것으로 요약된다. 따라서 *U. macrocarpa* Hance는 제약 산업에 사용될 수 있는 가능성을 나타내었다.