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쥐의 파라쿼트 중독 모델에서 설포라판의 영향

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- Abstract -

Sulforaphane Post-treatment Had No Protective Effects in Paraquat-intoxicated Rats

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Purpose: Sulforaphane is a naturally-occurring isothiocyanate abundant in broccoli. It has been suggested as a promising antioxidant. In this study, the therapeutic effect of sulforaphane in paraquat intoxication was investigated.

Methods: Paraquat was administered via the tail vein, after which sulforaphane or a vehicle (4% DMSO) was administered intraperitoneally 15 minutes after paraquat administration. Histological injury, lipid peroxidation, plasma cytokine (IL-6, IL-10), and nitric oxide were measured. In addition, the effect of sulforaphane on survival in paraquat-intoxication was observed.

Results: Regarding histological injury, lipid peroxidation, and plasma cytokine and nitric-oxide response, sulforaphane administration showed no protective effects in paraquat-intoxicated rats. Rather, it increased mortality (log rank p=0.03) and caused lipid peroxidation, as well as plasma cytokine and nitric-oxide production, to be increased.

Conclusion: Sulforaphane had no therapeutic effect on paraquat-intoxicated rats; rather, it increased mortality.

Key Words: Paraquat, Sulforaphane, Lipid peroxidation

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I. Introduction

Paraquat is a widely used and extremely effective herbicide. However, it is highly toxic to human and animals and causes one of the most clinically significant intoxications. The mechanism of paraquat intoxication includes generation of free radicals and oxidative stress. It undergoes redox cyclinginside the cell and subsequently generates reactive oxygen species (ROS).(1,2) These ROS interacts with lipids of cell membrane resulting in the destruction of organelles, inevitably leading to cell death.(3) Several antioxidants have been investigated, but effective treatment has not been currently confirmed yet.(4,5)

Sulforaphane [1-isothiocyanate-(4R)-(methylsulfinyl) butane] is a dietary isothiocyanate which is synthesized from a precursor found in cruciferous vegetables of the genus Brassica such as broccoli, kale, and cabbage. It has been widely studied for its anticancer properties.(6) However, beyond its anticancer properties, antioxidant effect has been reported in several acute models of tissue damage associated to oxidative stress. It includes intestinal ischemia/reperfusion injury,(7) ischemic cardiac injury,(8) ischemic brain injury,(9) organophosphate intoxication,(10) and traumatic brain injury.(11) In those studies, sulforaphanetreatment resulted in induction of several antioxidant enzymes of nuclear factor E2 related factor 2(Nrf2) dependent pathways including NADPH quinone oxidoreductase, glutathione-S-transferase, and heme oxygenase-1.(12,13) Considering the mechanism of paraquat intoxication, antioxidant properties of sulforaphane might be beneficial in paraquat poisoning.

Thus, the objective of this study is to examine the therapeutic effects of sulforaphane in paraquat intoxication in rats.

II. Materials and Methods

1. Experimental animal

This study was approved by the Animal Care and Use Committee of our institute and was conducted in accordance with National Institute of Health guidelines. Male Sprague-Dawley rats (Orient Bio, Seoul, Korea) weighing 300~350 g were used for experiment. The rats were housed in a controlled environment with free access to food and water for one week before the experiment.

2. Experimental procedure

Two sets of experiment were performed to examine the therapeutic effects of sulforaphane in paraquat intoxication.

1) Effects of sulforaphane in paraquat-induced lung and liver injury

To investigate the anti-oxidant effect of sulforaphane in paraquat-induced tissue (lung and liver) injury, tissue harvest study was performed.To induce paraquat intoxication, 50 mg/Kg of paraquat (Sigma-Aldrich Chemical Co. Ltd., St. Louis, MO, USA), which was suspended in 0.9% NaCl solution with final concentration of 50 mg/mL, was administered via tail vein. After injection of paraquat, rats were randomly allocated into one of two groups (Control group (n=7) vs. sulforaphane group (n=7)). Sulforaphane group was received D, L-sulforaphane (Sigma-Aldrich Chemical Co. Ltd., St. Louis, MO, USA) at 5 mg/Kg in 4% DMSO intraperitoneally 15 minutes after paraquat administration. Control group was administered same amount of 4% DMSO intraperitoneally. The dose of sulforaphane was chosen by previous published studies,(9,11) which was proven to have protective effects in rat model of oxidative stress. For tissue harvest, rats were sacrificed at 6 hour after paraquat injection. Rats were anesthetized with intramuscular injection of zoletil (50 mg/Kg) and xylazine(10 mg/Kg). After anesthesia, laparotomy was performed, and blood sample was obtained from abdominal aorta. Blood was centrifuged at 3000 rpm for 15 minutes at 4°C, and separated plasma was stored at -70°C for subsequent assays. Lung and liver tissues were harvested and fixed in 4% formalin and some of tissues were stored in -70°C liquid nitrogen.

2) Effect of sulforaphane on mortality in paraquat-intoxicated rats

Same experimental procedures were performed for survival study. After administration of study drug or vehicle, 50 mL/Kg of 0.9% NaCl solution was injected subcutaneously for fluid resuscitation and then rats were observed for survival for 48 hours.

3. Measurements

1) Histological injury score

Formalin-fixed lung and liver tissues were embedded in paraffin and sectioned at 4 ㎛. For histological examination, the sections were deparaffinized and stained with hematoxylin and eosin. The extent of lung injury was scored by a board-certified pathologist blinded to the groups according to alveolar congestion, hemorrhage, infiltration of neutrophils in the air spaces or vessel walls and the hyaline membrane formation.(14) The severity of each category was scored from 0(minimal) to 4(maximal) and the sum of each score was calculated ranging from 0 to 16. The histological liver injury was scored with a following morphologic criteria(15): spotty necrosis, capsular inflammation, portal inflammation, ballooning degeneration, and steatosis. Spotty necrosis was graded and scored as follows; 0=none, 1=one focus or less per 10×objective, 2=two to four foci per 10×objective, 3=five to ten foci per 10×objective, 4=more then ten foci per 10×objective. Capsular inflammation was graded and scored in each×10 area following magnification for the presence of capsular inflammation as follows; 0=none, 1=capsular inflammation in 1×10 magnification area; 2=capsular inflammation in 2×10 magnification areas, 3=capsular inflammation in 3×10 magnification areas. Portal inflammation was scored as follows; 0=none, 1=mild, some, or all portal areas, 2=moderate, some, or all portal areas, 3=marked, all portal areas. Ballooning degeneration was scored as follows; 0=none, 1=ballooning degeneration in one third of hepatic lobule, 2=ballooning degeneration in two thirds of hepatic lobule, 3=ballooning degeneration in all parts of hepatic lobule. Steatosis was scored as follows; 0=none, 1=〈30% hepatocyte containing fat, 2=30%~70% hepatocyte containing fat, 3=〉70% hepatocyte containing fat.The liver injury severity score was ranged 0(none) to 16(severe).

2) Lipid peroxidation of lung and liver tissues

Thiobarbituric acid reactive substance (TBARS) is a marker of lipid peroxidation and was measured by QuantiChrom TBARS Assay kit (DTBA-1000, BioAssay systems, Hayward, CA, USA).The concentration of TBARS was presented as ㎛M malondialdehyde (MDA) equivalents.

3) Plasma cytokines and nitric oxide concentration

Plasma cytokines (Interleukin-6(IL-6) and 10(IL-10)) were measured using commercial enzyme linked immunosorbent assay kit (R&D system, Minneapolis, MN, USA). Nitric oxide (NO) is rapidly deactivated to nitrie (NO_2^-) and nitrate (NO_3^-) by oxidation in plasma. Plasma NO was measured with commercial kit for nitrite/nitrate using Griess reaction (R&D system, Minneapolis, MN, USA).

4. Statistical analysis

Data were expressed as mean±standard error of mean. Student's t-test and Mann-Whitney U test were performed as appropriate. Survival rates were compared by Kaplan-Meier log-rank test. Statistical analyses were conducted using STATA 10.0/IC (Stata Corp LP. Texas, USA). A p-value〈0.05 was considered to be statistically significant.

III. Results

1. Effects of sulforaphane in paraquat-induced tissue injury

For tissue harvest study, 14 rats were studied (each group has 7 rats).

1) Histological injury score

Lung and liver histological injury was compared. Both injury scores were not significantly different; rather sulforaphane group had a tendency of increased injury score (Fig. 1).

2) Lipid peroxidation

Paraquat generates ROS and the ROS interacts with the lipid of cell membrane. Thus, we measured the products of lipid peroxidation in liver and lung tissues and it showed that sulforaphane post-treatment did not decrease the production of thiobarbituric acid reactive substances in lung (sulforaphane group=5.1±0.1 μg MDA equivalent/mg tissue; control

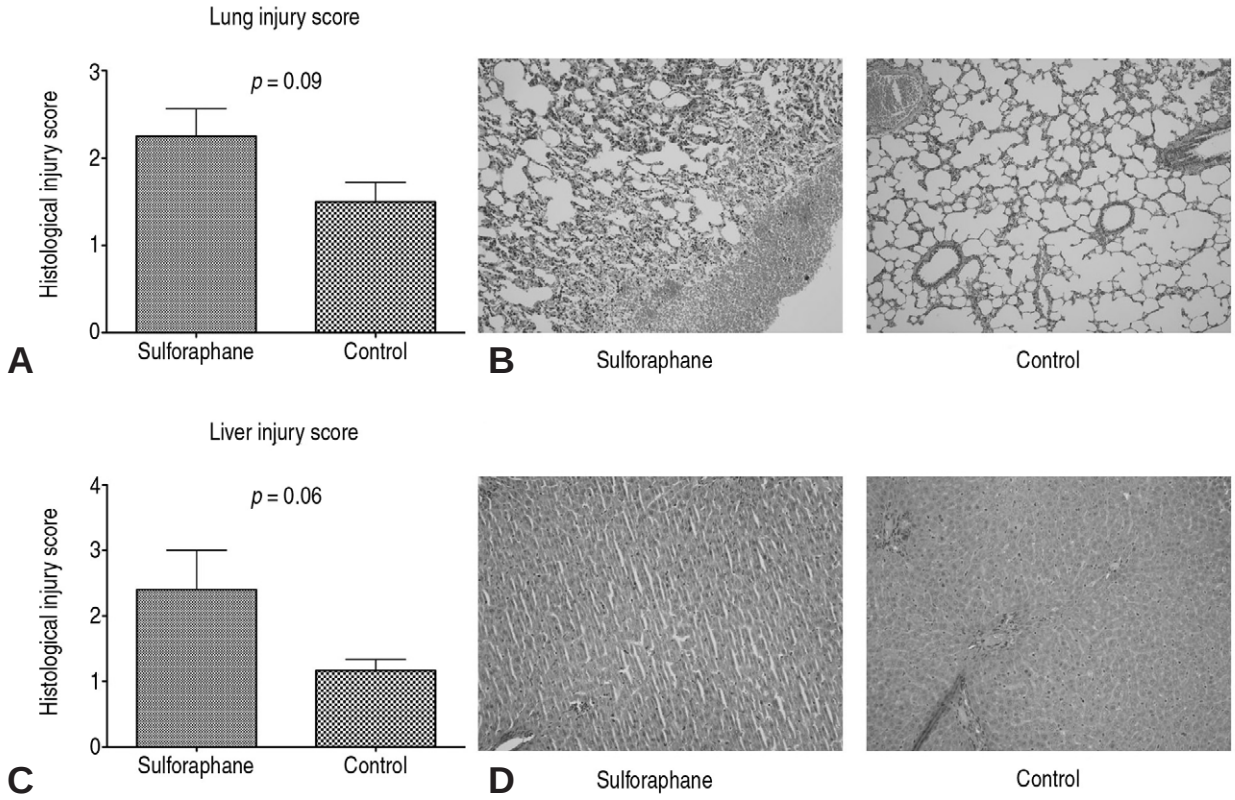

Fig. 1. Histological injury (**A**) Lung histological injury score (**B**) Representative photos of paraquat induced lung injury. In sulforaphane group, alveolar congestion and hemorrhage were shown. However, mild congestion was shown in control group. (**C**) Liver histological injury score (**D**) Representative photos of paraquatinduced liver injury. In sulforaphane group, spotty necrosis (arrow head) and diffuse attenuation of cellular membrane and widening of cytoplasm (=ballooning) were shown. However, in control group, microvesicular fatty change (arrow) and vivid cellular membrane with normal cytoplasmic volume (=normally appearance of hepatocytes) were shown.

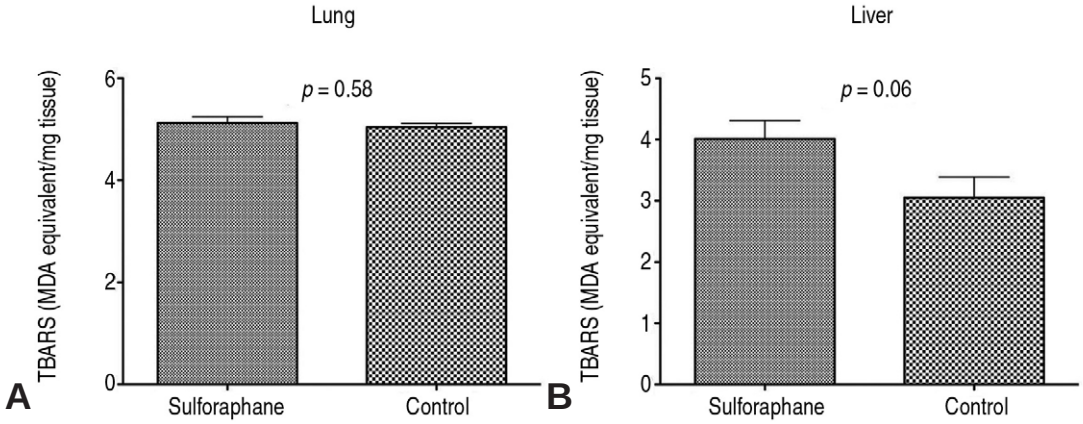

Fig. 2. Lipid peroxidation (**A**) Thiobarbituric acid reactive substances in lung tissue (**B**) Thiobarbituric acid reactive substances in liver tissue

group=5.0±0.1 μg MDA equivalent/mg tissue, p=0.58, Fig 2A). and liver (sulforaphane group=4.0±0.3 μg MDA equivalent/mg tissue; control group=3.1±0.3 μg MDA equivalent/mg tissue, p=0.06, Fig. 2B).

3) Plasma cytokines and nitric oxide

To evaluate the effect of sulforaphane on the inflammatory cytokine expression, IL-6 and IL-10 were measured in plasma. IL-6 and IL-10 were not significantly different between groups (p=0.05 for IL-6, p=0.73 for IL-10, Fig. 3A, B).

Plasma nitrite/nitrate concentration was not significantly different between groups (p=0.05, Fig. 3C)

2. Effect of sulforaphane on mortality in paraquat-intoxicated rats

During 48 hours of observation, all rats were dead in sulforaphane group and 2 rats were dead in control group (5/5 (100%) vs. 2/5 (40%), log rank p=0.03, Fig. 4).

IV. Discussion

The results of this study demonstrate that sulforaphane post-treatment did not show beneficial effect in paraquat intoxication. Rather sulforaphane increased mortality, and lipid peroxidation, inflammatory cytokine expression and NO production was not significantly different but showed a tendency to be increased in sulforaphane group.

Initially sulforaphane has been introduced as a chemopreventive agent. Sulforaphane is formed by enzymatic hydroxylation of precursor glucoraphanin (4-methylsulfinylbutyl glucosinolate), which is generally found in high concentration in broccoli. In cancer research, it has been known that it induces cell cycle arrest, apoptosis and inhibition of angio-

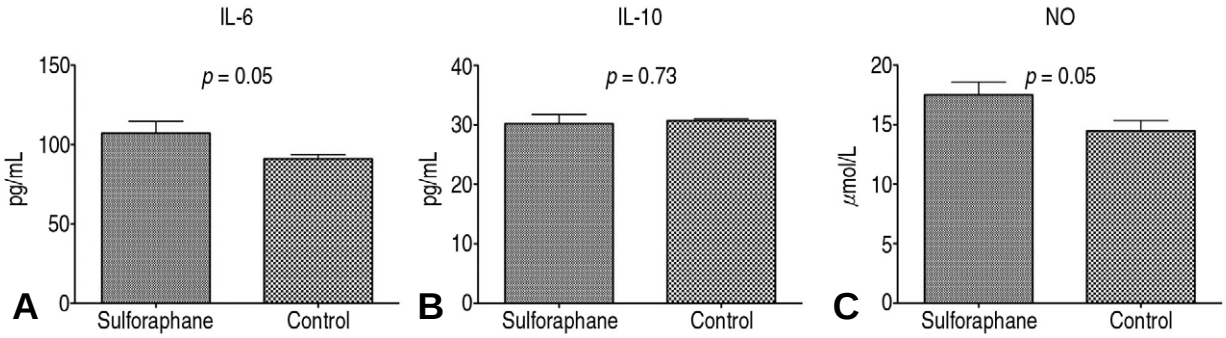

Fig. 3. Plasma cytokine and nitric oxide production (**A**) Plasma interleukin-6 (**B**) Plasma interleukin-10 (**C**) plasma nitric oxide production

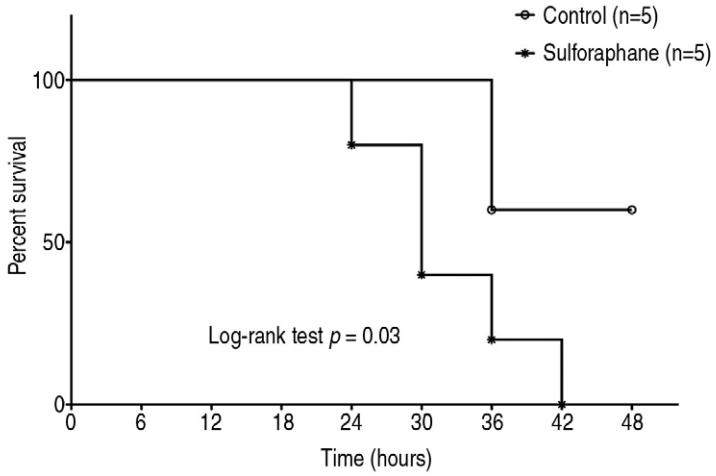

Fig. 4. Effect of sulforaphane on mortality in paraquat-intoxicated rats

genesis, and generation of ROS has been proposed as a preceding mechanism.(16,17) Sulforaphane also has been known to induce a phase II detoxification enzymes and it promotes a disruption of nuclear factor E2-factor related factor (Nrf2)-Kelch-like ECH-associated protein (Keap1) interactions and mitogen-activated protein kinase activation.(18,19) Thus, Nrf2 increases gene expression like NQO1, HO-1, glutathione reductase and glutathione peroxidase via antioxidant response element (ARE). In addition, sulforaphane showed immunomodulatory effects in some experiments using inflammatory cells stimulated by lipopolysaccharide.(20-24) Thus, sulforaphane has been suggested as a promising anti-oxidant and immunomodulatory agent to treat oxidative and inflammatory stress like ischemia/reperfusion injury and inflammatory disease.(25,26)

In this study, lipid peroxidation was not significantly different between groups, especially in lung tissue. Insignificant differences of lipid peroxidation of liver and lung tissues might be caused by early harvest of tissues. In survival study, the first mortality was observed in 24 hours after paraquat administration in sulforaphane group. Thus, tissues harvested at 6 hours could not be significantly different. Although the lipid peroxidation was not significantly different, TBARS production showed a tendency to be increased in liver tissue. In lung tissue, lipid peroxidation was similar between both groups. However, sulforaphane increased mortality. As mentioned above, sulforaphane increases the generation of ROS in cancer cell, and shows anti-cancer effect. In normal cells, there is a report that sulforaphane increased ROS production.(27) Payen et al. studied the effects of sulforaphane on ROS-related Multidrug Resistance-associated Protein 2 expression in primary hepatocytes. In their study, 20 μM sulforaphane/2% DMSO (v/v) increased ROS production from primary hepatocytes. Thus, pro-oxidant effect of sulforaphane might increase mortality in our study. This is similar to the effect of vitamin C in paraquat intoxication. Vitamin C has been known as a promising anti-oxidant. Thus, there have been trials to evaluate the therapeutic effect of vitamin C in paraquat intoxication. However, vitamin C showed both anti-oxidant and pro-oxidant effects according to experimental settings.(28) Iron chelation has been suggested to have the role in deciding the pro-oxidant or anti-oxidant effect of vitamin C.(29) However, there is no report about the role of iron chelation in the anti-oxidant effect of sulforaphane. Further study will be needed to elucidate the pro-oxidant or anti-oxidant effects of sulforaphane.

In this study, we administered sulforaphane 15 minutes after paraquat injection for testing the therapeutic effects. However, we did not find any protective effects of sulforaphane in paraquat intoxication model of rats. There are many reports that sulforaphane reduces oxidative stress in various animal models. However, sulforaphane was administered before the onset of injury in most studies and a few reports the beneficial effect of post-treatment, especially in brain injury model. Zhao et al. reported that sulforaphane post-treatment reduced infarct volume in cerebral ischemia model of rats.(9) In their study, sulforaphane was administered intraperitoneally, 15 minutes after the onset of ischemia and this was before the reperfusion. Another study, which investigated the role of Nrf2 signaling in traumatic brain injury (TBI) model, showed that sulforaphane 15 minutes post-treatment after brain injury significantly reduced oxidative damage and increased antioxidant enzyme HO-1 and NQO1.(11) In those studies, sulforaphane induced Nrf2-dependent gene expression and those might have roles in reducing brain injury. Although brain injury might include oxidative stress in these models, the period of maximum oxidative stress might be different from paraquat intoxication. Paraquat is rapidly distributed to tissues after absorption and starts to produce ROS within minutes. Thus, sulforaphane post-treatment does not have enough time to generate endogenous anti-oxidants. If sulforaphane will be administered before the paraquat intoxication, this may have protective effects. To clarify this, further studies will be needed.

There are several limitations in our study. First, we did not conclude whether sulforaphane in paraquat intoxication might be beneficial or harmful by this small animal study. In this study, we did not

reach the significant result on the effects of sulforaphane in paraquat intoxication except mortality. This might be derived from early harvest of tissues. However, sulforaphane post-treatment significantly increased mortality during 48-hour observation period. Second, we did not evaluate the effects of sulforaphane pre-treatment and other dose of sulforaphane on paraquat intoxication. This study was designed to evaluate the therapeutic potential of sulforaphane in paraquat intoxication. Thus, pre-treatment was not considered initially and the dose of sulforaphane chosen in this study was derived by previous studies, which showed antioxidant effects of sulforaphane post-treatment. Thus, further study will be needed. Third, we did not evaluate the sole effects of sulforaphane. Sham-operated animal experiments will be needed to clarify whether sulforaphane has anti-oxidant or pro-oxidant effects in this model. Fourth, anti-oxidants like NQO1, HO-1 or glutathione and ROS were not measured. Thus, the pro-oxidant effects of sulforaphane could not explain increased mortality in sulforaphane group. However, TBARS production showed increased tendency in sulforaphane group, although the difference was not significant between groups. Further study will be needed.

V. Conclusion

In this study, sulforaphane post-treatment did not show protective effects in paraquat intoxication of rats. Rather, it increased mortality.

VI. Acknowledgements

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All authors have no conflict of interest

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