J. KOREAN SOC. FOOD NUTR.
18(3) 239~246

화분의 영양생화학적 연구

-Aniline이 유도한 간 독성에 미치는 진달래 화분의 영향-

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Nutritional Biochemical Study on the Pollen Load

-Effect of Azalea Pollen on the Aniline-induced Hepatotoxicity-

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요 약

진달래 화분의 해독 작용을 연구할 목적으로 aniline을 model 약물로 하여 간 조직에 대한 병태생리학적 동태를 대사 효소의 활성과 관련지어 검토하였다. 진달래 화분 각 추출물을 투여한 군이 대조군에 비해 간 aniline hydroxylase의 활성이 증가되었으며, 진달래 화분 물 추출물 투여에 의한 간 aniline hydroxylase 활성 증가를 동력학적인 측면에서 관찰하였을 때 Km치는 변하지 않았으나 Vmax가 증가하는 점으로 보아 본 효소의 단백 합성이 촉진될 결과라고 사료된다. 또한 aniline만 투여한 군에 비해 진달래 화분 각 추출물을 투여한 실험군이 aniline의 혈중 농도와 간 손상을 현저히 감소시켰고, 이러한 작용은 물 추출물에서 가장 강하게 나타났다. 이로써 진달래 화분 투여에 의한 aniline의 간독성 예방 효과는 진달래 화분 추출물에 의한 간 aniline hydroxylase의 활성증가와 관련되어 나타난 것으로 사료되어진다.

Introduction

Numerous compounds when taken into the body are not directly conjugated but instead must first be metabolically transformed to yield a suitable acceptor for conjugation.[1-3] Initial reactions involved in the early metabolism of xenobiotics have classically been termed phase I reactions and encompass oxidative, reductive, and hydrolytic modifications.[4-6] Among the phase I reactions aniline hydroxylase is known as one of the enzymes that catalyze oxidation.[7-10]

Pollen is collected by the honeybee to provide a source of protein, carbohydrate, vitamins and minerals for developing bee larvae, and has been used a food source as well as a food supplement since the time of Hippocrates.[11] The chemical composition of pollen has already been the object of several investigations, and numerous classes of chemical substances of it has been isolated or identified.[12-14] The general tonic properties of pollen are at the basis of the therapeutic application in the case of general devitality and convalescent patients. However, although the effectiveness of pollen

* "This work was supported by the research grant from The Ministry of The Education in 1988.

on the patients with prostatic and urethral conditions, liver injury, atherosclerosis, diabetes mellitus, and hyperlipidemia was proved by many investigators,[15-20] the possible preventive effect of azalea pollen on any disease state has scarcely studied.

In the previous report, it was recognized that treatment with water extract of azalea pollen increaes the activity of hepatic aniline hydroxylase which catalyze the oxidation of aniline analog[21].

In the present study, it was undertaken to elucidate the preventive effect of each azalea pollen extract on the pathophysiological phenomenon including the tissue histological and biochemical changes produced by aniline as toxic substance.

Materials and Methods

Chemicals.

The chemicals were obtained from the following sources : Aniline and p-aminophenol from Wako Pure Chemical Co. : NADPH(Type I) and bovine serum albumin from Sigma Chemical Co. : trichloroacetic acid from Junsei Chemical Co.. All other reagents were of reagent grade commercially available.

Animals and Treatment.

Male ICR-mice weighing 20 to 25g were used for all studies. They were fed food and water *ad libitum* until the start of the expriment. Mice were randomly divided in four groups, and each group was treated with either saline or azalea pollen extract(ether, water or butanol extract)[21] at 10mg per kg per day, intraperitoneally, for 5 days. 12hr after the last injection of pollen extract, aniline(110 mg/kg) was treated intraperitoneally for 2 days before mice were killed. Animals were allowed free access to food and water but deprived of food for 16hr prior to sacrifice.

Preparation of Hepatic Microsomal Fraction.

Animals were killed by exsanguination from inferior vena cava. Liver was exhaustively perfused with cold 0.15M NaCl solution through the portal vein until uniformly pale and quickly excised. After mincing, the piece of liver was homogenized in 4 vol. of 0.25M sucrose. Each homogenate was centrifused at 10,000xg for 20 min.. The resulting supernatant was centrifuged at 105,000xg for 60 min. and the resultant microsomal fraction resuspended in 0.25M sucrose and used.

Enzymatic Determinations.

Serum aminotransferase(ALT, AST) activities were measured by the method of Reitman and Frrankel[22] using a commercial kit. Hepatic microsomal aniline hydroxylase activity was measured by the method of Bidlack et al[23]. with aniline and NADPH as substrates. In brief, the enzyme activity was determined by measuring the amount of formed p-aminophenol (nmoles/mg protein/min.). Under the assay conditions used, the initial rates of p-aminophenol formation demonstrated linear function with time and protein concentration. Protein was determined by Lowry method with bovine serum albumin as standard[24].

Measurement of Aniline Level in Serum.

After removal of protein, aniline in serum was determined using a 10% trichloroacetic acid by the method of Norwitz et al.[25] Aniline level was expressed as mg/mℓ of serum.

Histological Assessment of Liver.

The liver was excised, fixed, dehydrated and paraffin-infiltrated. Liver sections were stained with hematoxylin and eosin[26].

Results

Effect of azalea pollen extracts on serum ami-

Table 1. Effect of azalea pollen extracts on the serum alanine and aspartate aminotransferase(ALT, AST) activities in aniline-treated mice

Treatment	Aminotransferase acitivity(Karmen unit/mℓ of serum) ALT	AST
Control	31.7± 7.3	56.8± 9.5
Aniline	130.8± 17.8***a)	143.9± 21.8**a)
Ether extract+Aniline	77.0± 15.5*a·b)	128.6± 25.5**a)
Butanol extract+Aniline	64.8± 10.0**b)	108.7± 24.5
Water extract+Aniline	43.0± 13.4**b)	90.0± 14.1

Mice were injected each azalea pollen extract(10mg/kg) i.p. daily for 5 days, and killed 24hr after the last dose of aniline(110mg/kg). The assay procedure was described in the experimental methods. Values are means ± S. E. for 5 animals. a) : significantly different from control(* ; $p<0.05$, ** ; $p<0.01$, *** ; $p<0.001$), b) : significantly different from aniline.

notransferase activities in the aniline-treated mice.

Blood samples for the determination of serum aminotransferase activities were collected 24hr after the administration of aniline. The results are summarized in Table 1 and show that the increase of serum ALT activity in aniline-treated group was 4.0 fold when compared to the control group. However, pretreatment of each azaelea pollen extract before the anilien injection has markedly improved the status of serum aminotransferase activities when compared to the aniline-treated group. The improved effect was greater in water extract-pretreated group than in any others.

Effect of azalea pollen extracts on the hepatic histological changes in the aniline-treated mice.

The hepatic toxicity of aniline was studied in mice pretreated with azalea pollen extracts. As shown in Fig. 1., the liver structure of animals treated with saline is normal. In mice treated with aniline, however, mild degeneration with inflammatory cell infiltration of hepatocytes around central vein is evident, but no inflammatory cell infiltration in ether extract-pretreated group. In mice pretreated with butanol extract, mild dilatation of sinusoids is seen, but no hepatocellular degeneration. Pretreatment with water extract before aniline treatment prevented the aniline-induced toxi-

Table 2. Effect of azalea pollen extracts on the hepatic microsomal aniline hydroxylase activity and serum aniline level in mice

Treatment	Aniline hydroxylase acitivity (nmoles/mg protein/min.)	Serum aniline level (mg/mℓ)
Control	0.82± 0.06	0.380± 0.026
Ether extract	0.84± 0.08	0.341± 0.029
Butanol extract	1.01± 0.06*	0.266± 0.023*
Water extract	1.15± 0.07**	0.220± 0.024**

Mice were injected each azalea pollen extract i.p. daily for 5 days, and killed 24hr after the last injection. Aniline was injected i.p. 60min. before sacrifice in the case of mesurement of aniline level. The assay procedure was described in the experimental methods. Values are means ± S. E. for 5 animals. significantly different from control(* ; $p<0.05$, ** ; $p<0.01$).

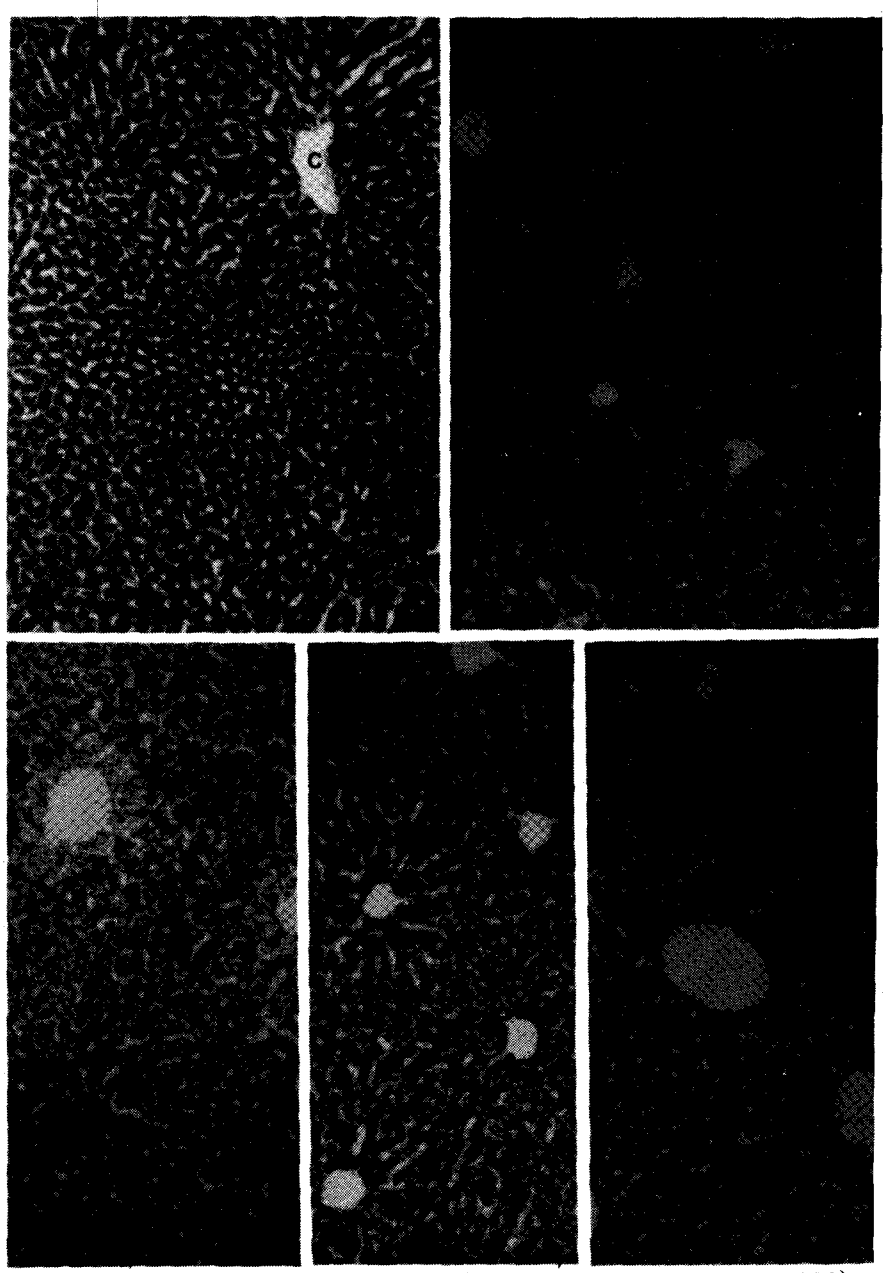

Fig. 1. Photomicrographs of hepatic tissue in mice.(Hematoxylin and Eosin stain, x100)
All conditions are the same as described in Table I.
A ; Liver specimen of control,
B ; Liver specimen of aniline-treated group,
C ; Liver specimen of ether extract and aniline-treated group.
D ; Liver specimen of butanol extract and aniline-treated group.
E ; Liver specimen of water extract and aniline-treated group.
(c ; central vein, p ; portal tract)

city, and hepatic structure is similar to control group.

Effect of azalea pollen extracts on the hepatic microsomal aniline hydroxylase activity, serum aniline level in mice.

Table 2. shows the increase of hepatic microsomal aniline hydroxylase activity, and the decrease of serum aniline level following repeated administration of azalea pollen extracts for 5 days. Futhermore, the enzyme activity was significantly elevated, and aniline level was strikingly decreased as compared to the control group when water extract was injected to mice.

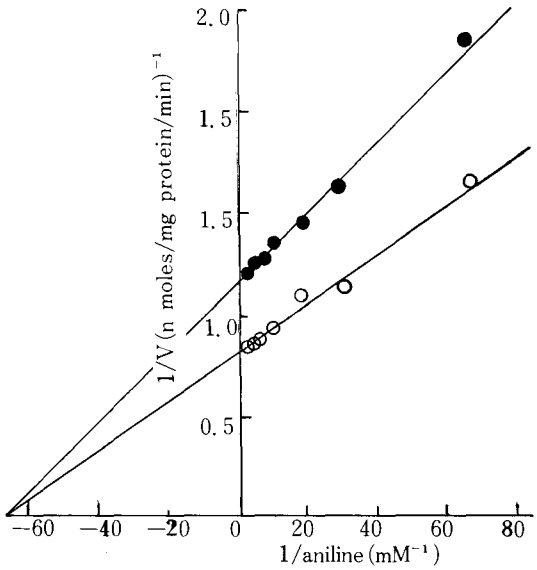

Fig. 2. Double reciprocal plots of the hepatic microsomal aniline hydroxylase activity as a function of varying concentrations of aniline at fixed level of NADPH. Mice received azalea pollen water extract i.p. daily for 5 days. The assay procedure was described in the experimental methods. Data points represent the mean of 3 experiments. Control ; ●, Azalea pollen water extract ; ○.

Effect of water extract on the kinetic properties of the hepatic microsomal aniline hydroxylase.

To study the effect of azalea pollen water extract on the kinetic parameters, initial rate of hepatic microsomal aniline hydroxylase activity was measured as a function of variable concentrations of aniline at a fixed concentration of NADPH (Fig. 2). Apparent Km value for aniline in microsomes in saline-treated mice was 15.7 μM ; respective Km in water extract-treated mice was 15.7 μM. There was no change in apparent Km values for aniline in the water extract-treated mice, compared to controls. When plotted on double reciprocal form, the Vmax value for aniline increased about 1.5 fold by the treatment of water extract.

Discussion

Since pollen is known to be used as a food supplement and a medicine, the understanding of its role in drug metabolism is essential for a complete description of hepatic dysfunction. Generally, hydroxylation is one of the major oxidation reactions involved in the metabolic conversion of xenobiotics to more water-soluble products.[6-8] The results of the present study have demonstrated that treatment with azalea pollen extracts prevented the aniline-induced hepatotoxicity. Futhermore, serum aminotransferase activities increased many fold in the aniline-treated group while no significant rise was observed in the water extract-pretreated group. This result indicated that biochemical changes caused by aniline may be prevented by the treatment with water extract. It was also observed that treatment with azalea pollen extracts prevents against aniline-induced histological changes. The preventive effect was greater in water extract-pretreated group than in any others. Thus, induction of hepatic aniline hydroxylase activity by water extract does not appear to depend upon the quantity of water extract, but upon the specific com-

pound in water extract. At present, it is not resolved the structure of compound that increases hepatic aniline hydorxylase activity. It is well known that aniline is metabolized primarily by hydorxylation. This metabolite is further metabolized and excreted in the urine as a p-aminophenol glucuronide[10]. The enzyme that catalyzes this reaction is known as UDP glucuronyltransferase.[27, 28] It was also observed that water extract treatment increased the activity of hepatic UDP-glucuronyltransferase(data not shown). The results of the present study confirm that treatment with water extract is effective in reducing the extent of aniline-induced liver injury in an animal model. The preventive effect seems to be due to altered aniline metabolism.

Previous studies with mice have demonstrated that treatment with azalea pollen extract increases the hepatic microsomal aniline hydroxylase activity.[21] The increasement of hepatic microsomal aniline hydroxylase activity can change either due to an alteration in the quantity of enzyme protein or due to catalytic activation of existing enzyme. To differentiate between these possiblilities, we determined the kinetic properties of hepatic microsomal aniline hydorxylase. The apparent Km value of the reaction was not significantly changed for aniline, whereas, the Vmax value increased with water extract treatment compared to control group. Thus, as phenobarbital[29], the characteristics of the increase in the enzyme activity, may result from a change in the quantity of enzyme proteins, rather than an activation of enzyme activities due to changes in the lipid environment or other factors[30].

In this study, it was indicated that the action mechanism of azalea pollen extract on the aniline level and hepatotoxicity may be associated with the modulation of the aniline metabolizing enzyme systems, but further research in this field is needed.

Abstract

Preventive effect of azalea pollen extracts against aniline-induced hepatic toxicity in mice was investigated in this experiment. When the biochemical and histological changes were measured, preventive effect was more striking by treatment with water extract. After treatment with azalea pollen extracts, hepatic microsomal aniline hydroxylase activity increased as compared to control. Whereas, aniline level in serum and liver significantly decreased. The Vmax value without affecting Km value increased by the water extract treatment, the results obtained suggest that the characteristics of increase in the aniline hydroxylase activity may include induction of enzyme proteins. These data indicate that the observed preventive effects of azalea pollen extracts against hepatotoxicity is due to the induction of aniline metabolizing enzyme.

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(Received May 13, 1989)