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Effect of Tungstate Supplemented Diet on the Toluene Metabolism in Rats

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Abstract

To evaluate an effect of oxygen free radical on the toluene metabolism, the rats were fed on a tungstate supplemented diet (0.75 g of tungstate included in 1 kg of standard diet) or a standard diet. To the present xanthine oxidase deficient animal model, toluene (0.15 ml/100 g of body weight) was injected and then the animals were sacrificed after 24 hrs to determine the toluene metabolizing enzyme activities and toluene metabolite, hippuric acid concentration. The increasing rate of urinary hippuric acid concentration was significantly ($p<0.01$) higher in tungstate fed animals than that in standard diet fed ones. Hepatic cytochrome P-450 contents were significantly higher ($p<0.01$) in tungstate fed animals than in standard diet fed ones. And tungstate fed animals showed a tendency of higher activities of benzylalcohol dehydrogenase while a significantly higher activities of benzaldehyde dehydrogenase ($p<0.01$) than standard diet fed animals. In conclusion, the more possibly reduced oxygen free radical in toluene-treated rats fed with a tungstate supplemented diet than in those fed with a standard diet would be responsible for the enhancement of toluene metabolism.

Key words: tungstate supplemented diet, toluene metabolism, oxygen free radical, rats

INTRODUCTION

Tungsten-rich diet blocks oxygen free radical toxicity through inactivation of xanthine oxidase (1). It has therefore been processed as an anti-diet for paraquat toxicity (2). And the superoxide free radical, O_2^- is generated in many biological reactions that reduce molecular oxygen (3). O_2^- is also produced during the action of several oxidative enzymes, i.e., xanthine oxidase, aldehyde oxidase, and dihydroorotic dehydrogenase (4). It is known that the main route of oxygen free radical generation can be an xanthine oxidase system (5). Such oxygen free radical enhances degradation of critical enzymes by the multicatalytic proteosome complex, raising havoc throughout the cell (6).

On the other hand, the toluene which is used mainly as a solvent in industry, led to toxic effect on the nervous (7-9), circulatory system (10,11), liver (12) etc., and the mechanism of action responsible for toluene toxicity has not been established, but may include metabolic activation and covalent binding of toluene metabolite to tissue macromolecules (13).

In experimental animals, side chain oxidation and ring hydroxylation are the two major routes of toluene metabolism by cytochrome P-450 (14). And then side chain oxidation of toluene produce benzylalcohol (15). Benzylalcohol is further oxidized by the sequential action of alcohol dehydrogenase and aldehyde dehydrogenase to produce first benzaldehyde and then benzoic acid (16). Benzoic acid is subsequently conjugated with glycine to produce hippuric acid (16) and it excreted into urine (17). Recently in our previous paper (18), metabolic rate of xenobiotics such as toluene is effected by its metabolizing enzyme activity. And the oxygen free radical as above mentioned may be responsible for inhibition of toluene metabolizing enzyme activity.

Based on this view, to clarify whether the oxygen free radical might have influence upon the toluene metabolism, we investigated the hepatic toluene metabolizing enzyme activities and urinary toluene metabolite, hippuric acid concentration in the xanthine oxidase deficient experimental animal model as fed tungtate supplemented diet.

MATERIALS AND METHODS

Animals and toluene treatment

Male Sprague-Dawley rats weighing about 100 g were fed on a tungstate supplemented or standard diet as described in Table 1 for one month, and intraperitoneally injected one dose of 0.3 ml toluene (50% in olive oil) per 100 g body weight.

Control rats were given olive oil only. Water was provided *ad libitum*. Rats were sacrificed 24 hrs after the injection. The animals were divided into 4 groups (tungstate fed animal, standard diet fed animal and each control; each containing 6 rats.

The animals were killed by exanguination of abdominal aorta. The liver was exhaustively perfused with cold 0.9% saline solution through the portal vein. The liver of rats was rapidly removed and homogenized in a ice-cold 0.25 M sucrose solution. Homogenates (20%, w/v) were centrifuged at 700 × g for 10 min. The supernatants obtained were spun at 10,000 × g for 30 min at 4°C. The postmitochondrial fraction was again

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Table 1. Composition of experimental diet (g/kg diet)

Ingredients	Standard diet	Tungstate diet
Casein	200	200
Corn starch	670	670
Corn oil	54	54
Vitamin A & D mixture[1)]	10	10
Vitamin E & K mixture[2)]	2	2
Water soluble vitamins[3)]	3	3
Vitamin B_{12}[4)]	1	1
Salt mixture[5)]	40	39.28
Na_2WO_4	-	0.72
α-Cellulose	20	20
	4190.9 kcal	4190.9 kcal

[1)]51,000 unit of vitamin A and 5,100 unit of vitamin D dissolved in 100 ml of corn oil.
[2)]5 g of α-tocopherol and 0.2 g of menadione dissolved in 200 ml of corn oil.
[3)]Contained (mg): choline chloride 2,000, thiamine hydrochloride 10, riboflavin 20, nicotinic acid 120, pyridoxine 10, Ca-panthothenate 100, biotin 0.05, folic acid 4, inositol 500 and *p*-amino benzoic acid 100.
[4)]5 mg of vitamin B_{12} dissolved in 500 ml of distilled water.
[5)]Contained (g); $CaCO_3$ 300, potassium phosphate dibasic 321.45 (standard diet) or 303.65 (tungstate diet), $MgSO_4$ 102, Ca-phosphate monobasic 75, NaCl 167.5, ferric citrate 27.5, KI 0.8, $ZnCl_2$ 0.25, $CuSO_4 \cdot 5H_2O$ 0.3, $MnSO_4$ 5, molybdic acid 0.2 (standard diet) or Na_2WO_4 18 (tungstate diet).

centrifuged at 105,000×g for 60 min and then the cytosolic supernatants and microsomal fractions were obtained.

On the other hand, 24 hr urine specimens were collected in metabolic gauge for the determination of hippuric acid concentration.

HPLC assay for urinary hippuric acid concentration

The amount of hippuric acid in urine was analysed by high performance liquid chromatography. Urine was pretreated by the method of Kubota et al. (19). 200 µl of urine was diluted 3-fold with acetonitrile, followed by centrifugation. 5 µl of supernatant was injected. A mixed solution of water-methanol-acetic acid (80:20:0.20, by volume) was used as eluent at a flow rate of 1 ml/min. Detection was done by UV detector at 235 nm. Concentration of hippuric acid was calculated based on the area of chromatogram with standard solution. Analysis of creatinine in the same sample was done by Butler method (20) using Jaffe reaction. Unit of hippuric acid was used as g/creatinine g.

Enzyme assay

Hepatic benzylalcohol or aldehyde dehydrogenase activity in the postmitochondrial fraction was determined by measuring formation of NADH using benzylalcohol or aldehyde, respectively as substrate, NAD as coenzyme according to the procedure of Bergmeyer (21). Enzyme unit was described as µmole of NADH/min/mg of protein.

Demonstration of xanthine substrate effect on the benzylalcohol or aldehyde dehydrogenase activity *in vitro*

For *in vitro* test of oxygen free radical effect on the benzylalcohol or aldehyde dehydrogenase activity, postmitochondrial supernatant (30 mg protein) was added to 100 µmole of xanthine and then preincubated for 30 min at 37°C. Using the enzyme specimen such as prepared, benzylalcohol or aldehyde dehyrodgenase activity was determined. The protein including in enzyme reaction mixture was assayed by the method of Lowry et al. (22).

Cytochrome P-450 content

The content of cytochrome P-450 was measured by difference spectrum with double beam spectrophotometer according to the method of Omura and Sato (23). The statistical significance of difference between values were analyzed by Student's t-test (24).

RESULTS AND DISCUSSION

The animal fed tungstate diet showed somewhat the tendency of increased rate of body weight compared with the standard diet group, but there were statistically no differences in body weight gains between two groups (Fig. 1). This result indicates that a felicitous quantity of tungstate included in a diet prepared in the present experiment would not attribute to hazardous to the animals.

Although a felicitous quantity of tungstate in the diet could not lead to toxicity, it causes depletion of xanthine oxidase (XO) activities in animals (25-27). In the present experiment conditions, the activities of hepatic XO of tungstate diet group was also remarkably dropped to 15% compared with standard diet group (Fig. 2).

In the present XO deficiency animal model, by the injection of toluene, the increasing rate of urinary hippuric acid concentration was significantly ($p<0.01$) higher to about 52% in tungstate-fed animals than that in standard diet group (Fig. 3). This suggests that tungstate may influence upon the enhancement of metabolism by facilitating of excretion of toluene metabolite, hippuric acid. Based on this view, to clarify the cause increasing urinary hippuric acid more in tungstate

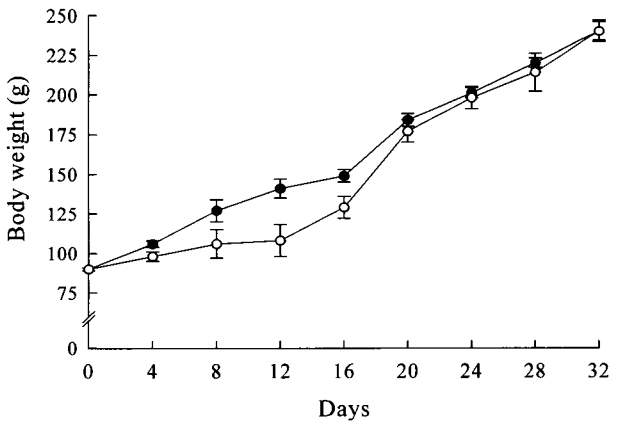

Fig. 1. One month weight gains in rats fed tungstate supplemented diet or standard diet. Each value is the mean±S.E. of 12 rats. ○—○: Standard diet, ●—●: Tungstate diet.

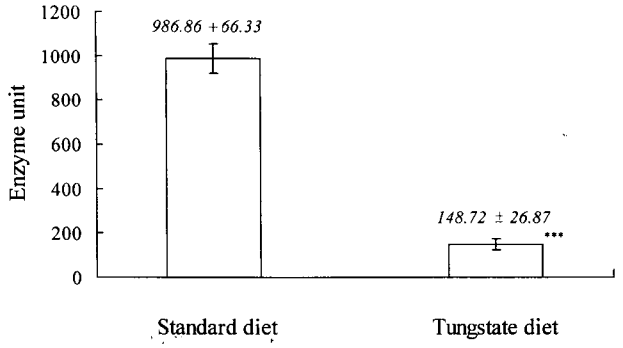

Fig. 2. Hepatic xanthine oxidase activities in rats fed a tungstate supplemented diet or standard diet. Each value represents the mean ±S.E. of 6 rats. ***Significantly different from the group fed a standard diet ($p<0.001$). Unit: nmoles uric acid formed/min/100 g body weight.

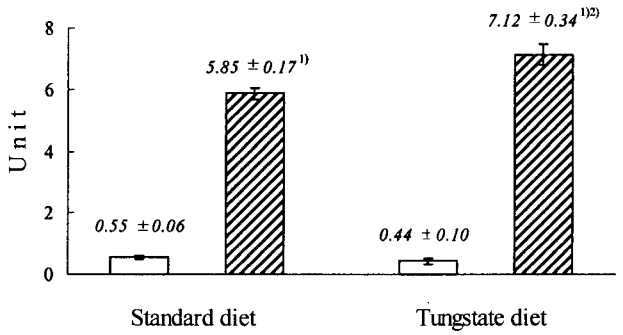

Fig. 3. Urinary hippuric acid concentration in toluene-treated rats fed a tungstate supplemented diet or standard diet. [1)]Significantly different from the control ($p<0.001$), [2)]Significantly different from the rats fed a standard diet treated with toluene ($p<0.01$). Other abbreviations are the same in Fig. 2. Unit: Hippuric acid g/creatinine g. □: Control, ▨: Toluene treated.

fed animals, we investigated the hepatic cytochrome P-450 content and some toluene metabolizing enzyme activities chiefly playing an important role in toluene metabolism (Table 2) (18).

Table 2 showed the hepatic cytochrome P-450 content and toluene metabolizing enzymes, benzylalcohol or aldehyde dehydrogenase activities in toluene-treated rats fed tungstate supplemented diet or standard diet. Hepatic cytochrome P-450 contents were significantly higher ($p<0.01$) in tungstate fed rats than standard diet fed ones. And tungstate fed animals showed a tendency of higher activities of benzylalcohol dehydrogenase and a significantly higher activities of benzaldehyde dehydrogenase ($p<0.01$) than standard diet fed animals.

Table 2. The hepatic cytochrome P-450 (CYP) content, benzylalcohol (BADH) and benzaldehyde (BALDH) dehydrogenase activities in rats fed tungstate supplemented diet or standard diet

Groups	Standard diet	Tungstate diet
CYP[2)]	0.35±0.01[1)]	0.40±0.01**
BADH[3)]	4.56±0.58	5.09±0.42
BALDH[4)]	5.12±0.29	6.59±0.25**

The assay procedure is described in experimental methods. [1)]Each value represents the mean±S.E. of 6 rats. **Significantly different from the standard diet fed group ($p<0.01$). Unit: [2)]nmoles/mg protein, [3,4)]μmoles NADH/mg protein/min.

Based on these results and the general facts (28) that increasing activity of enzyme acting on its substrate can influence upon the enhancement of producing metabolite, the decreasing activity of toluene metabolizing enzyme in standard diet-fed animals may be responsible for the cause of low urinary hippuric acid concentration. Paradoxically speaking, it is likely that the increased toluene metabolizing enzyme activity in tungstate-fed animals may be caused to less effect on oxygen free radical compared with the standard diet fed animal.

These facts may be supported that oxygen free radicals can be produced chiefly through the route of xanthine oxidase enzyme system (5), and such as oxygen free radical is known to inactivate an enzyme activity (6). Therefore, the cause of increasing activity of toluene-metabolizing enzyme in tungstate-fed animals may be less effective on the production of oxygen free radical. Furthermore, in the present experiment, the cause of increasing activity of the enzymes such as benzylalcohol or aldehyde dehydrogenase *in vitro* was clarified by the determination of these enzyme activities in liver extract in the presence of xanthine as well as in the absence of this substance to observe the effect on oxygen free radical.

As shown in Fig. 4, benzylalcohol dehydrogenase was inhibited by 13% and aldehyde dehydrogenase was also inhibited by 40% compared with controls respectively. These findings confirmed that oxygen free radical generated from xanthine substrate can inhibit the benzylalcohol or aldehyde dehydrogenase activity.

In conclusion, the more reduced oxygen free radical in toluene-treated rats fed tungstate than in those fed a standard diet would be responsible for the enhancement of toluene metabolism.

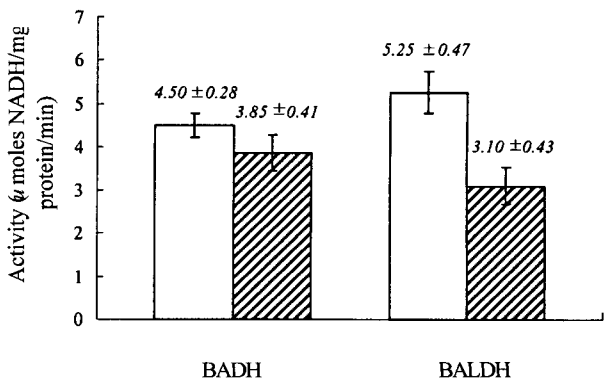

Fig. 4. Effect of xanthine on the activities of hepatic benzylalcohol dehydrogenase (BADH) and benzaldehyde dehydrogenase (BALDH) *in vitro*. Each value represents the mean±S.E. of 5 experiments. □: Control, ▨: Toluene treated.

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