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# Effects of Hydroxybrazilin on Glutathione Depletion Induced by $BrCCl_3$ and Menadione in Cultured Rat Hepatocytes

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## ABSTRACT

In this study we investigated the effect of hydroxybrazilin on glutathione depletion induced by $BrCCl_3$ and menadione in cultured hepatocytes to understand the cellular mechanisms of hepatoprotective effect of hydroxybrazilin. Hydroxybrazilin alone had no effect on total glutathione level and the ratio of reduced glutathione/total glutathione (GSH/(GSSG+GSH)). $BrCCl_3$ dramatically decreased total glutathione level and hydroxybrazilin significantly prevented glutathion depletion by $BrCCl_3$. The ratio of GSH/(GSSG+GSH) was also decreased by $BrCCl_3$ and recovered by hydroxybrazilin treatment. Menadione decreased total glutathione level and the ratio of GSH/(GSSG+GSH) but hydroxybrazilin showed no significant effects on menadione-induced glutathione depletion. These data suggest that hydroxybrazilin might prevent the hepatotoxicity induced by chemical-derived radicals but not the toxicity linked with oxidative stress.

**Key words** : Hydroxybrazilin, Bromotrichloromethane, Menadione, Cultured hepatocyte, glutathione

## INTRODUCTION

Glutathione is the most important and widely distributed nonprotein thiol in living systems, which plays a major role in many redox and detoxification reactions in the liver[1]; reduced form of glutathione (GSH) traps the electrophillic metabolites of some toxic chemicals and prevents their binding to hepatic proteins and enzymes[2-4] and the conjugation of the chemical with glutathione usually results in the formation of a nontoxic, water-soluble metabolites that is easily excreted. Nearly all of the glutathione is present as reduced form (GSH) and the amount of oxidized form of glutathione disulfide (GSSG), is very much lower. Redox status of these thiols is maintained by intracelluar glutathione reductase and NADPH.[5]

In recent years, the redox transition of glu-

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tathione has been reported to affect or modulate a variety of cellular process, including enzyme activity[6] and the transport of small solutes and ions,[7] especially calcium.[8] And disturbances in intracellular glutathione homeostasis induced by hepatotoxins were associated with changes in cell surface structure and preceded cell death.[9] It has also been reported that lowering of the intracellular glutathione level by the addition of diethyl maleate promoted the ADP · $Fe^{3+}$-induced cellular lipid peroxidation (malondialdehyde production), and the formation of conjugated dienes.[10]

According to our previous studies,[11-13] some γ-pyranoidal natural dyes such as brazilin and hydroxybrazilin have hepatoprotective effects. They lowered sALT and sAST levels in the $CCl_4$-treated mice and reduced lipidperoxidation in ethanol- and $CCl_4$-treated rats. In the present study, we studed the effects of hydroxybrazilin on the $BrCCl_3$- and menadione-induced GSH depletion in cultured rat hepatocytes to investigate the cellular hepatoprotective mechanisms of by hydroxybrazilin.

## MATERIALS AND METHODS

### 1. Materials

SD male rats (200~250g) were obtained from Laboratory Animal Center, Seoul National University, housed 2~4 per cage, and allowed foods and water *ad libitum*. $BrCCl_3$ was obtained from Wako Chemical Co., Japan. Hydroxybrazilin was obtained from Merck Co., Germany. Other reagents were obtained from Sigma Chemical Co., USA.

### 2. Monolayer culture of hepatocytes

Hepatocytes were isolated from SD male rats by a collagenase perfusion technique as described by Berry and Friend.[14] The isolated hepatocytes were inoculated onto collagen coated dishes in $CO_2$ incubator and then, monolayer was formed within 4 hours. This monolayer hepatocytes were cultured for 24 hours in William's E medium supplemented with 5% FBS.

### 3. Determination of intracellular glutathione

The medium was replaced with serum free William's E medium containing hydroxybrazilin (1~100 μM). $BrCCl_3$ (0.2 μM/ml) or menadione (100 μM) was added directly to the culture medium and incubated for 30 or 60 minutes, respectively. At the end of incubation, the cells were washed with ice cold phosphate buffered saline. The washed hepatocytes were collected with 0.5% picric acid (1 ml) into microcentrifuge tube. After centrifugation, the supernatant was withdrawn for the determination of tatal glutathione and oxidized form of glutathione. To determine the oxidized glutathione, reduced glutathione was derivatized with 2-vinylpyridine. The amount of glutathione was determined by DTNB (5, 5'-dithiobis-2-nitrobezoic acid)-GSSG (oxidized glutathione) reductase recycling method.[15] Reduced form of glutathione (GSH) was calculated from the difference between the total and the oxidized glutathione.

All statistical comparisons were performed using a Student t-test. Differences were considered statistically significant at $p<0.05$.

## RESULTS AND DISCUSSION

Hydroxybrazilin, a γ-pyranoidal natural dye, is known to have various biological activities including hepatoprotective effects. In order to clarify cellular hepatoprotective mechanisms of hydroxybrazilin, we introduced the monolayer cultured hepatocyte system, and investigated the effects on the glutathione depletion induced by hepatotoxins such as $BrCCl_3$ and menadione. For the appplication of hydroxybrazilin to in vitro hepatocyte system, the

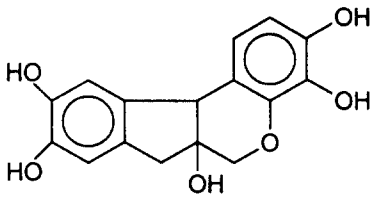


**Fig. 1.** Structure of hydroxybrazilin.

**Table 1.** Effects of hydroxybrazilin on glutathione level and GSH/(GSH+GSSG) ratio in normal hepatocytes.

| Groups | GSH+GSSG (nmol glutathione /3×10⁶cells) | GSH | GSH/(GSH+GSSG) (% value) |
|---|---|---|---|
| Normal | 101±5 | 89±5 | 88.1% |
| $10^{-6}$M Hrx | 107±3 | 94±1 | 87.9% |
| $10^{-4}$M Hrx | 105±4 | 92±4 | 87.6% |

Hrx : hydroxybrazilin
Values are the mean±SE of three separate experiments

suitable concentration of hydroxybrazilin which does not affect the cellular glutathione level should be found. As shown in table 1, 100 μM and 10 μM of hydroxybrazilin had no effect on total glutathione level and GSH/(GSH+GSSG) ratio. hydroxybrazilin was not cytotoxic to monolayer cultured hepatocytes at these or lower concentrations.

Carbontetrachloride ($CCl_4$) has been widely used for the investigation of chemical-induced hepatotoxic mechanism. But $CCl_4$ was not evaluated as a suitable hepatotoxin for the in vitro study, especially in cultured rat hepatocytes, in which the level of cytochrome P-450 is markedly lowerd. In the present study, $BrCCl_3$ was used as a hepatotoxin instead of $CCl_4$ because $BrCCl_3$ metabolized to $\cdot CCl_3$ radical more easily than $CCl_4$.[16] Trichloromethane radical ($\cdot CCl_3$) attacks membrane lipids, cellular proteins and DNA. Subsequently it causes lipid peroxidation and cellular damage leading to cell death. BrCCl3 treatment on the monolayer cultured hepatocytes showed depletion of total glutathione level (Table 2).

**Table 2.** Effects of hydroxybrazilin and vitamine E on glutathione level and GSH/(GSH+GSSG) ratio in $BrCCl_3$-treated hepatocytes.

| Groups | GSH+GSSG (nmol glutathione /3×10⁶cells) | GSH | GSH/(GSH+GSSG) (% value) |
|---|---|---|---|
| Normal | 129± 6 | 123±7 | 95.3% |
| $BrCCl_3$ | 15± 3* | 5±1* | 33.3% |
| $BrCCl_3$+$10^{-6}$M Hrx | 13± 4 | 6±4 | 46.2% |
| $BrCCl_3$+$10^{-5}$M Hrx | 15± 8 | 9±7 | 60.0% |
| $BrCCl_3$+$10^{-4}$M Hrx | 61±10# | 48±6# | 78.7% |
| $BrCCl_3$+$10^{-4}$M Vit.E | 39± 8# | 27±6# | 69.2% |

$BrCCl_3$ $1.5\times10^{-3}$M
Hrx : hydroxybrazilin, Vit E : Vitamine E
Values are the mean±SE of three separate experiments
* ; Significantly different from normal control group
# ; Significantly different from $BrCCl_3$-treated group

**Table 3.** Effects of hydroxybrazilin on glutathione levels and GSH/(GSH+GSSG) ratios in $BrCCl_3$- and menadione-treated hepatocytes.

| Groups | GSH+GSSG (nmol glutathione /3×10⁶cells) | GSH | GSH/(GSH+GSSG) (% value) |
|---|---|---|---|
| Normal | 100±12 | 90±11 | 90.0% |
| $BrCCl_3$ | 16± 3* | 5± 1* | 31.3% |
| $BrCCl_3$+$10^{-4}$M Hrx | 57± 4# | 44± 8# | 77.2% |
| Menadione | 57± 5* | 30± 2* | 52.6% |
| Menadione+$10^{-4}$M Hrx | 45± 3 | 21± 3 | 46.7% |

$BrCCl_3$ $1.5\times10^{-3}$M, Menadione $10^{-4}$M
Hrx : hydroxybrazilin
Values are the mean±SE of three separate experiments
* ; Signiicantly different from normal control group
# ; Signiicantly different from $BrCCl_3$-treated group

The major loss of glutathione has been thought to be the result of the decrease of the reduced form of glutathione during $BrCCl_3$ metabolism. The highest concentration of hydroxybrazilin (100 μM) recovered the glutathione loss in the monolayer cultured haptocytes. And this concentration of hydroxybrazilin was more effective than that of vitamine E. Vitamine E, a well known antioxidant, scavenges free radicals and the hydroxyl group on aromatic ring of vitamine E forms chroma-

noxy or phenoxy radicals.[17] Similarly, it can be proposed that the hydroxyl group on aromatic ring of hydroxybrazilin may donate hydrogen to $\cdot CCl_3$ radical, thus detoxifying the $BrCCl_3$ toxicity.

With this concentration, we introduced another radical generating system to compare the effect of hydroxybrazilin on intracellular glutathione loss. Incubation of cultured hepatocytes with menadione (200 μM) for 1 hour resulted in a significant decrease in intracellular reduced form of glutathione but reduction in the total equivalents of glutathione was not comparable to that of $BrCCl_3$ treatment (Table 3). In isolated hepatocytes, a marked decrease of intracellular glutathione (GSH) level has been observed during the metabolism of menadione.[18] This is known to be related to the formation of $\cdot O_2^-$ and $H_2O_2$ caused by redox cycling of the quinone, in particular to the metabolism of $H_2O_2$ by the selenoprotein glutathione peroxidase, an enzyme which removes peroxides at the expense of GSH.[19] And these processes cause a oxidative stress in the cell. GSH can directly form a conjugate with menadione which is subsquently excreted from the cell[8] but the menadione-conjugate is not thought to give a major contribution to the glutathione loss.[19] As shown in table 3, 100 μM of hydroxybrazilin had no effect on menadion-intoxicated hepatocytes. Thus, it was thought that hydroxybrazilin could not trap the free radicals formed from the metabolism of menadione. These data suggest that hydroxybrazilin might prevent the hepatotoxicity induced by chemical-derived radicals but not the toxicity linked with oxidative stress.

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